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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Apoptotic cell-death in the brain is a common feature in a variety of neurodegenerative diseases and following exposure to neurotoxins. We hypothesize that certain components of the signaling pathways activated by pathophysiological stimuli might be shared and could serve as targets for the development of therapeutic approaches. In our application, we proposed to compare the signaling pathways activated by four different apoptotic stimuli using cultures of rat cerebellar granule neurons with the goal of identifying common signaling molecules. During the first three years, our goal was to use one of these apoptotic stimuli - potassium (K+) deprivation - and examine the role of four different apoptosis-regulatory molecules. We have now confirmed that NF-κB is a molecule central to neuronal survival and have obtained substantial information regarding the mechanism by which NF-κB activity is regulated in neurons undergoing apoptosis. We show that NF-κB activity is also affected by the neurotoxins, methylmercury and 6-hydroxydopamine. We have gathered evidence indicating that p38-β activation is necessary for neuronal survival. We show that Akt is necessary for the survival promoting effect of IGF-1, but not of K+. In research that has just been published, we show that contrary to common belief, Akt lies downstream and not upstream of NF-κB activation.

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INTRODUCTION

Apoptosis is a cell-suicide process that is required for the normal development of the nervous system. Aberrant and inappropriately regulated apoptosis can, however, lead to undesirable neuronal loss such as that seen in certain neurodegenerative diseases. Apoptosis can also be induced in various neuronal populations by chemical and biological neurotoxins. The intracellular pathways by which these different physiological and pathophysiological stimuli cause neuronal death has not been characterized. We hypothesize that certain components of the signaling pathways activated by different apoptotic stimuli might be shared. Our expectation is that once identified, such molecules could serve as ideal targets for the development of approaches to protect or treat individuals against the actions of neurotoxic agents. In our grant application, we had proposed to compare the signaling pathways activated by four different apoptosis-inducing stimuli using cultures of cerebellar granule neurons from the rat brain with the goal of identifying common molecular components. Specifically, we proposed to induce apoptosis in neuronal cultures by (i) potassium (K+)-deprivation, (ii) treatment with the environmental neurotoxin, methyl mercury, (ii) treatment with b-amyloid protein (βAP) accumulation, which has been implicated in Alzheimer's disease, and (iv) overstimulation with the neurotransmitter, glutamate. We proposed to examine the involvement of four signaling molecules - NF-κB, p38 MAP kinase, caspases, and mGluR4 - in the regulation of cell-death by the different apoptotis-inducing stimuli. In a request for supplemental funding that was approved last year, we had proposed to extent our investigation to another well-studied apoptosis-regulatory molecule - the serine-threonine kinase, Akt.

BODY

During the first three years we had proposed to examine whether four molecules - NF-κB, p38 MAP kinase, caspases, and mGluR4 - are causally involved in the regulation of low K+ (LK)-mediated neuronal apoptosis. As reported in our second annual report that was submitted last year, we obtained evidence that NF-κB and p38 MAP kinase were involved in the regulation of LK-mediated apoptosis. During the past year, we have substantially strengthened our data regarding NF-κB. Furthermore, we have obtained interesting data on the role of Akt in the survival of granule neurons and its relationship to NF-κB. Our results are described below:

NF-κB

As indicated in our report last year, we have fully completed the experiments we had proposed to do concerning the role of NF-κB in low K+ (LK)-induced apoptosis. Our results, which were published in the Journal of Neurochemistry (Koulich et al., 2000) showed that NF-κB is necessary for neuronal survival, and that the activity of this transcription factor is substantially reduced in neurons primed to undergo apoptosis. Although activation of NF-κB is generally regulated at the level of nuclear translocation or in some cases phosphorylation of p65, these mechanisms are not utilized in granule neurons. We have found that in neurons, NF-κB activity is regulated by the interaction of p65 the transcriptional coactivator, CREB-binding protein (CBP). The association between p65 and CBP is reduced in neurons induced to undergo apoptosis by LK-treatment. This decrease in NF-κB transcriptional activity is accompanied by a reduction in the interaction between p65 and CBP an alteration that is accompanied by hyperphosporylation of CBP. LK-induced CBP hyperphosphorylation can be mimicked by inhibitors of protein phosphatase 2A (PP2A) and PP2A-like phosphatases such as okadaic acid

and cantharidin, which also causes a reduction in p65-CBP association. In addition, treatment with these inhibitors induces cell-death. Treatment with high concentrations of the broad-spectrum kinase inhibitor staurosporine prevents LK-mediated CBP hyperphosphorylation and inhibits cell death. In vitro kinase assays using GST-CBP fusion proteins map the LK-regulated site of phosphorylation to a region spanning residues 1662 - 1840 of CBP. Our results are consistent with possibility that LK-induced apoptosis is triggered by CBP hyperphosphorylation, an alteration that causes the dissociation of CBP and NF-κB. The results described above have been submitted for publication to the Journal of Neurochemistry, a copy of which is attached to this report. We have been informed that the manuscript is accepted subject to rather minor revisions. Because the manuscript has been provided we have not included the data here.

In accordance with our proposed plans, we have begun to examine if reduction in NF-κB activity represents an alteration that is caused by different neurotoxic stimuli. We have begun preliminary experiments using methylmercury and another neurotoxic agent, 6-hydroxydopamine and analyzed their effect of the DNA-binding activity of NF-κB using EMSA. As shown in Figure 1, in a preliminary experiment treatment with both these neurotoxic agents reduced NF-κB even more than that caused by LK treatment.

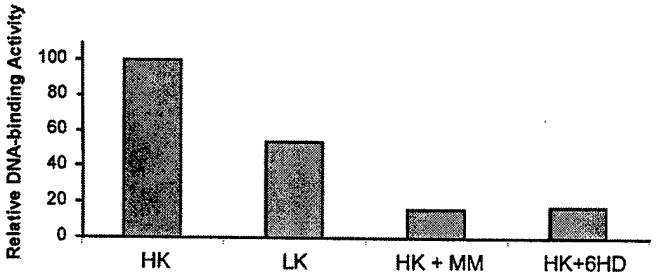

Fig. 1: EMSA assay of NF-kB binding activity. Neuronal cutures were treated for 6h with LK, HK, HK + methylmercury (MM), or HK + 6-hydroxy dopamine (6-HD). Nuclear extracts were prepared and used in EMSA assays with a NF-κB probe. Relative binding activity was qualified by densitometric analysis.

p38 MAP kinase

As we reported in our last report, pharmacological inhibition of p38 MAP kinase protects granule neurons from LK-mediated apoptosis. Moreover, while the expression of total p38-α and p38-β is the same under HK and LK conditions, the phosphorylation of p38-β is reduced following LK-treatment. p38-α phosphorylation, on the other hand, remains unchanged. In preliminary experiments we examined the effect of overexpression of wild-type and dominant-negative p38-α and p38-β on neuronal viability. As reported last year, we find that overexpression of p38-α induces apoptotic death in cultures kept in HK-medium whereas similar overexpression of p38-β reduced LK-induced apoptosis. Taken together, our results suggest that p38-α is proapoptotic while p38-β is antiapoptotic in neurons. p38-β serves to counterbalance the apoptotic effects of p38-α. Reduced p38-β activity (as seen after LK-treatment) therefore causes apoptosis by permitting p38-α to exert its apoptotic effects.

Given that p38 MAP kinases are involved in the regulation of LK-induced apoptosis, we have begun to examine whether it might similarly affect apoptosis induced by the various neurotoxins. Preliminary results (not shown) suggest that treatment with methylmercury causes a reduction in p38-β phosphorylation.

Akt-related results

In our request for a supplement to our grant, we had proposed to investigate the role of Akt in the regulation of neuronal survival. As we have reported in a recent publication, Akt is

activated by all four survival factors in our system - HK, IGF-1, cyclic AMP, and lithium. During the past few months we have used a newly available pharmacological inhibitor of Akt, ML9, to look at the necessity for Akt in the survival effects of the four factors. As shown in Fig. 2A , treatment with ML9 blocks the survival effects of IGF-1 and of lithium, but has no effect on survival by HK or cAMP.

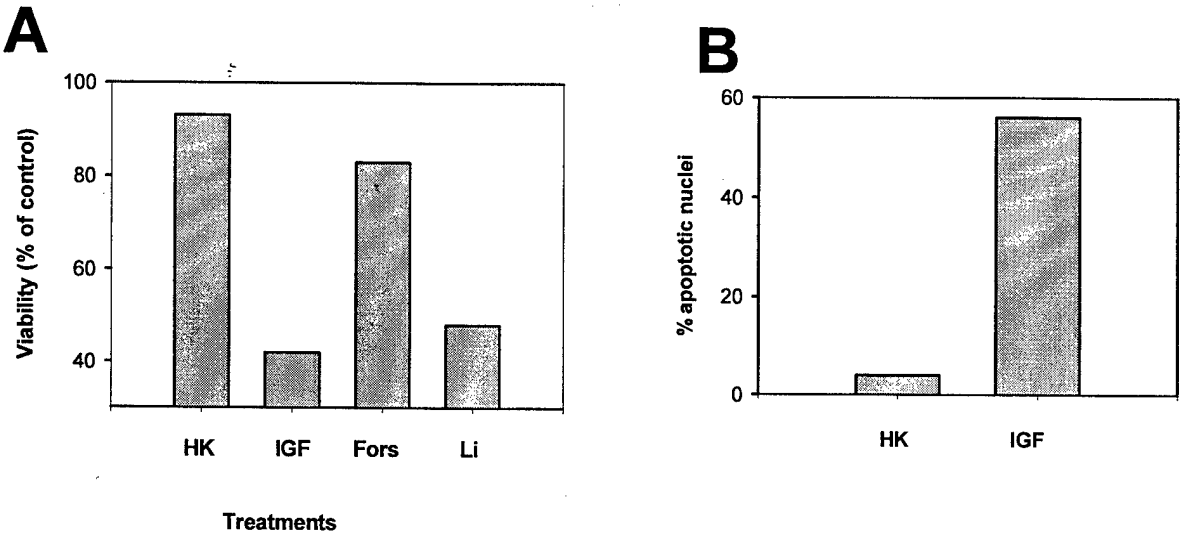

Fig. 2: Effects of a pharmacological inhibitor of Akt and dn-Akt expression on neuronal viability.
A. Effect of ML-9 on neuronal viability. Neurons were treated with HK, IGF-1, forskolin (to increase cAMP), or lithium in the absence or presence of 10um ML-9. Viability was assayed 24h later using the MTT assay. Viability with ML-9 treatment for each survival promoting agent is expressed as percentage of viability in its absence using the same survival factor.
B. Effect of dn-Akt expression. Cultures were infected with an adenoviral vector expressing HA-tagged dn-Akt and then switched to medium containing HK or IGF-1. The proportion of infected neurons (HA-positive) displaying apoptotic nuclei (condensed or fragmented as judged by DAPI-staining) was quantified.

Since pharmacological inhibitors are not always specific, we have begun to confirm these results using adenoviral vectors expressing dominant-negative (dn) forms of Akt. As shown in Fig. 2B, we have found that Akt is necessary for the survival-promoting effect of IGF-1. The survival effects of HK on the other hand, is not affected by dn-Akt. The results obtained using adenoviral vectors are consistent with those reported last year using plasmid. Since adenoviral vector infect with high efficiency, we will use it in our future studies. We are currently looking at whether the effects of cAMP and lithium are also inhibited by expression of dn-Akt. At least one study has shown recently that neuronal survival by cAMP does not require Akt[1], while another has suggested that lithium utilizes Akt[2]. Neuronal survival may therefore be maintained by Akt-dependent and independent pathways. It deserves mention that although all four survival factors activate Akt, they do so with different patterns of Akt phosphorylation. We have recently reported that while treatment with IGF-1 induces phosphorylation at both Thr308 and Ser473, HK and cAMP stimulate Akt phosphorylation at Thr308 only and phosphorylation by lithium occurs only at Ser473[3]. Taken together, our results raise the possibility that phosphorylation at Ser473 is critical for the survival-promoting effect of Akt.

Akt and NF-κB are both important for the survival effects of IGF-1. Using a variety of culture systems, it has been shown that Akt lies upstream of NF-κB in pathways in which both signaling molecules are activated. In our request for a supplement we had provided preliminary evidence that in neurons, NF-κB lied upstream rather than downstream of Akt. Specifically, we

showed results of a preliminary experiment in which treatment of neurons with the NF-κB inhibitor, SN-50, blocked Akt activation. In our request for a supplement we proposed to determine whether in fact, Akt lied downstream of NF-κB. We proposed to do this in initially in cell-lines and subsequently confirm the results in neurons. Over the past year we have rigorously confirmed that Akt is a downstream target of NF-κB. Using NIH3T3 cells we showed that the stimulation of Akt by the NF-κB activators, TNFα and lipopolysaccharide (LPS) can be detected only after IκB-α degradation (an essential step in NF-κB activation) occurs. The nuclear translocation of p65 and increased DNA-binding activity of NF-κB also precede Akt phosphorylation. Treatment with NF-κB inhibitors such as SN50 and TPCK, blocks TNF-induced Akt activation in cell-lines and Akt induction by IGF-1 in neurons. Finally, overexpression of p65 / RelA leads to Akt phosphorylation in the absence of extracellular stimulatory factors, while overexpression of IκB-α reduces Akt phosphorylation below basal levels. Interestingly, in addition to stimulating the phosphorylation of Akt, overexpression of p65 causes an increase in the expression of Akt mRNA and protein. Our results have been published in a paper that has just appeared in the Journal of Biological Chemistry[4]. Rather than providing the data here, we have attached a copy of our publication.

In summary, the results we have obtained over the last three years have shown that NF-κB (and possibly p38-β) is important for neuronal survival. LK treatment leads to a reduction of that NF-κB activity. We are now embarking on the second phase of our proposed research and examining if other neurotoxic stimuli induce similar alterations. Preliminary results (see Fig. 1) using methylmercury are consistent with the idea that reduced activity of NF-κB (and possibly p38-β) ισ necessary for the apoptotic activity of different neurotoxins. If so, we would have succeeded in our goal of identifying a convergent point in the signaling pathways affected by different neurotoxins. We have discovered that Akt is important for effects of some, but not all survival-promoting factors. In the signaling pathway activated by IGF-1 and other growth factors / cytokines, Akt is likely to be a downstream target of NF-κB.

KEY RESEARCH ACCOMPLISHMENTS

- Reduced activity of NF-κB is seen during apoptosis induced by LK and by two neurotoxins-methylmercury and 6-hudroxydopamine. These modifications could be a common signal by which different neurotoxins cause apoptosis in neurons.
- Elevated NF-κB activity in neurons observed under survival-promoting conditions is mediated by its interaction with CBP, which in turn is regulated by the status of CBP phosphorylation.
- Contrary to several recent reports, at least in some cases NF-κB is likely to lie upstream of Akt.

REPORTABLE OUTCOMES

- **Manuscripts:**

(a) Kumari S, Liu X, Nguyen T, Zhang X, D'Mello SR. (2001) Distinct phosphorylation patterns underlie Akt activation by different survival factors in neurons. Mol Brain Res. 96, 157-62.
(b) Meng F, Liu L, Chin PC, D'Mello SR. (2002) Akt Is a Downstream Target of NF-kappa B. J Biol Chem. 277, 29674-80.

(c) Yalcin A, Koulich E, Mohamed S, Liu L, D'Mello SR. Apoptosis in cerebellar granule neurons is associated with reduced interaction between CBP and NF-κB (Submitted, J. Neurochem.)

- **Abstracts:**

(a) Koulich E and D'Mello, SR. Apoptosis in cerebellar granule neurons is associated with CBP hyperphosphorylation and reduced interaction between CBP and NF-κB. Soc.Neurosci. Abst.
(b) Yalcin A, Koulich E, Mohamed S, D'Mello SR. Apoptosis in cerebellar granule neurons is associated with reduced interaction between CBP and NF-κB (Submitted for Soc. Neurosci. meeting in Orlando, FL)

- **Funding:**

Based on the results obtained from the project, we submitted a R01 grant application to the NIH titled "Signaling pathways regulating neuronal survival". The grant received a percentile score of 2.3 and was funded with a starting December 2001.

- **Training:**

Funds from the grant and the supplement were used in the training of postdoctoral fellows in the lab - Fanyin Meng and Salah Mohamed. Dr. Mohamed left the lab in February 2002 and was replaced with another postdoctoral fellow - Li Liu. In addition, funds have been used to support a new technician, Jamie Rhodes and during the summer graduate students - Paul Chin, Kyle Johnson, and Asligul Yalcin. Both students and the postdoctoral fellows have gained tremendously from the training made possible by the grant and are making substantial research contributions. Dr. Meng is the first author on a paper published in the Journal of Biological Chemistry recently, while Asligul is the first author on one that has been accepted (subject to revision) in the Journal of Neurochemistry.

CONCLUSIONS

Our results have led further insight into the mechanisms underlying apoptosis in neurons from the brain. Our search for molecules that are affected by different neurotoxic stimuli and has led to the identification of at least one viable candidate. Identification of such important molecules would be an important step in the development of therapeutic strategies aimed at preventing neuronal loss after exposure to neurotoxins.

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Modification of Statement of Work

In our grant we had proposed to examine the role of specific intracellular signaling molecules in the regulation of neuronal apoptosis induced by 4 distinct cell death-inducing stimuli. Initially (Aim 1), the role of the signaling molecules would be examined in neuronal cultures induced to die by LK-treatment. The results obtained using LK would be compared with signaling activated by three other neurotoxic stimuli (Aim 2). The goal was to identify common signaling molecules that are affected by all four cell death-inducing stimuli. The rationale was that if such common signaling molecules could be identified, they would serve as ideal targets for the development of therapeutic strategies against the actions of neurotoxic military threat agents and also in the treatment of neurodegenerative diseases such as Parkinson's disease. While we have made substantial progress on the DOD-funded project that had led to the publication of three scientific reports, we wish to modify the statement of work. The following are the reasons:

(a) We had proposed to use methylmercury, β-amyloid protein and glutamate as neurotoxic stimuli. At the time the proposal was submitted, we had not used these agents and as indicated in the proposal, our experimental plan was based on work published by other laboratories describing the apoptotic effects of these agents on cerebellar neuron cultures. While methylmercury treatment and glutamate do in fact induce neurodegeneration in cerebellar cultures, despite a substantial amount of effort we have been unable to reproduce the published results showing that β-AP can induce apoptosis in cerebellar cultures.

(b) The signaling molecules that we had proposed to study were NF-κB, p38 MAP kinase, mGluR4, and caspases. While we have confirmed that NF-κB and p38 MAP kinase are important in regulating neuronal apoptosis (unpublished work and our three papers listed above deal with this), we [1] (and other labs[2, 3]) have shown that LK-induced apoptosis in granule neurons occurs by a caspase-independent mechanism. Caspases, therefore, cannot represent a convergent point utilized by the different neurotoxic stimuli. Furthermore, we have found that treatment with mGluR4 agonists also do not reproducibly prevent LK-induced apoptosis.

Our goal will continue to be to identify apoptosis-regulatory molecules that are affected by different neurotoxic stimuli. Because of the reasons outlined above (which are based on work we have done recently), we would like to make the following changes -

1. We would like to drop β-AP from the list of neurotoxins. We would like to replace them with 6-hydroxydopamine (6HDA) and 1-methyl-4-phenylpyridinium (MPP+). We have confirmed the results of others[7,8] and found that both these neurotoxins induce cell death in cultured granule neurons [~80% and ~35% cell death, for 6HDA (40uM) and MPP+ (150uM), respectively]. It deserves mention that treatment of animals with 6HDA and MPP+ are known to cause Parkinson's like neuropathology. The signaling pathways activated by these neurotoxins will be studied, as outlined in our original proposal for the other neurotoxins.

2. Because of their inability to affect potassium-deprivation induced apoptosis, we would like to cease working on caspases and mGluR4. In view of our results indicating the significance of NF-κB, p38 MAP kinase to the regulation of neuronal apoptosis, we would like to focus on these two molecules in our future work.

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Short communication

Distinct phosphorylation patterns underlie Akt activation by different survival factors in neurons

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Accepted 6 September 2001

Abstract

The survival of cultured cerebellar granule neurons can be maintained by depolarizing levels of potassium (high K^+, HK), insulin-like growth factor (IGF-1), cyclic AMP or lithium. We examined the possibility that the signaling pathways activated by these different factors converge and that Akt might represent such a point of convergence. Consistent with this possibility, we find that Akt is phosphorylated and activated by all four survival factors. The pattern of Akt phosphorylation induced by the four survival factors, however, shows differences. While IGF-1 induces phosphorylation of Akt at both Ser473 and Thr308, HK and cyclic AMP stimulate phosphorylation at Thr308 only. Lithium increases phosphorylation at Ser473 but not at Thr308. Our results are consistent with the possibility that Akt is a central component of different survival-promoting pathways in granule neurons. The different phosphorylation patterns, however, point to a previously unappreciated complexity in the regulation of Akt activity in neurons. Finally, we provide evidence indicating that SGK, a kinase that is structurally related to Akt, is also activated by the four survival factors.

Keywords: Phosphorylation pattern; Akt; Survival factor; Cerebellar granule neuron; Cyclic AMP; Lithium

Apoptosis is a cell-suicide program that is necessary for the normal development of the nervous system (reviewed in Refs. [14,18,37,52]). Deregulation of the apoptotic program results in aberrant cell-death which is believed to occur in several neurodegenerative pathologies (reviewed in Refs. [37,40]). The intracellular mechanisms by which apoptosis is inhibited and neuronal survival maintained are presently unclear although much insight has been gained using cultures of primary neurons. Cultures of cerebellar granule neurons represent one such system. The survival of these neurons can be maintained by depolarizing levels of extracellular potassium (K^+ [24]). Switching of granule neuron cultures from a high K^+ (HK) medium to one with low K^+ (LK) induces apoptosis [15]. In addition to HK, several other factors/agents have survival-promoting activity on these neurons including insulin-like growth factor, cyclic AMP, lithium and the calcium-ATPase inhibitor, thapsigargin [15–17,34]. Interestingly, HK, cyclic AMP, and thapsigargin can also prevent apoptosis of peripheral sympathetic neurons which are normally dependent on nerve growth factor for their survival [21,33,42,44,45]. Similarly, the in vitro survival of other central and peripheral neuronal types and of differentiated neuronal PC12 cell-line can be supported by HK and cAMP, in addition to specific growth factors [25,29,45].

While growth factors such as IGF-1 and NGF activate receptor tyrosine kinases and promote neuronal survival by a phosphatidylinositide-3 kinase (PI-3K)-dependent pathway [10,17,19,20,26,36], HK-treatment causes membrane depolarization resulting in the influx of calcium through voltage-gated channels which is necessary for HK-mediated survival [23,30]. In cerebellar granule neurons, survival by HK occurs via a PI-3K-independent pathway [17,20], although this conclusion has been contradicted by some studies [36,47]. Neuronal survival by cyclic AMP on the other hand, requires neither elevated intracellular calcium nor PI-3K activation [10,37,38]. Although affecting distinct cell-surface molecules, the signaling pathways

Abbreviations: HK/LK, high/low potassium; IGF-1, insulin-like growth factor, PI-3k, phosphatidylinositide-3 kinase; SGK, serum-glucocorticoid kinase

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activated by these different survival promoting factors may converge on common downstream molecules. If so, such molecules would be ideal targets for the development of therapeutic approaches aimed at curing or preventing neuronal degeneration seen in a number of neuropathological conditions.

One molecule that may serve as a convergent point of different survival-promoting signaling pathways is Akt, a 60-kDa serine/threonine kinase which can be activated by a variety of growth factors including IGF-1 and NGF (reviewed in Refs. [1,9,28]). Activation of Akt has been shown to inhibit apoptosis, and thus promote survival, in many different cell-types and in response to several different stimuli. Growth factor-mediated activation of Akt is dependent on PI-3K activity and occurs via phosphorylation of two residues — Ser473 and Thr308 [1,9,28]. Mutational analysis has shown that while Thr308 is sufficient to activate Akt, both residues are required for maximal activation. In contrast to growth factor-mediated stimulation, however, HK treatment of the NG108 cell-line activates Akt in a PI-3K-independent pathway involving calcium-calmodulin kinase kinase (CamKK; [51]). Similarly, cyclic AMP may activate Akt in a PI-3K-independent manner [22,46]. Once activated Akt is thought to maintain cell-survival by inhibiting the actions of proapoptotic molecules such as the bcl-2-related protein, Bad [11,12], caspase-9 [7], the Forkhead transcription factor [5], and the IκB-phosphorylating kinase IKK [41,43].

In this study, we have used cultured cerebellar granule neurons to examine the role of Akt in the survival effects of four different factors — HK, IGF-1, cAMP, and lithium. We show that all four survival factors activate Akt. Interestingly, however, there are differences in the pattern by which these different survival factors phosphorylate Akt. Furthermore, SGK a kinase related to Akt is also phosphorylated by the different survival factors.

Unless specified otherwise, all chemicals and reagents were obtained from Sigma (St. Louis, MO). Antibodies to Akt were obtained from Cell Signaling Technologies (Beverly, MA). Recombinant IGF-1 was purchased from Roche Biochemicals (Indianapolis, IN).

Granule neuron cultures were obtained from dissociated cerebella of 7–8-day-old rats as described previously [15]. Cells were plated in Basal Eagle's medium with Earles salts (BME) supplemented with 10% fetal calf serum (FCS), 25 mM KCl, 2 mM glutamine (Gibco-BRL), and 100 μg/ml gentamycin on dishes coated with poly-L-lysine in 24-well dishes at a density 0.5×10^6 cells/well or 2.5×10^7 cells/100 mm dish. Cytosine arabinofuranoside (10 μM) was added to the culture medium 18–22 h after plating to prevent replication of non-neuronal cells. Unless indicated otherwise, cultures were maintained for 6–7 days prior to experimental treatments with IGF-1 or pharmacological agents. For this, the cells were rinsed once and then maintained in low K^+ medium (serum-free BME medium, 5 mM KCl) with or without the agents, or in the case of control cultures, in high K^+ medium (serum-free BME medium, supplemented with 20 mM KCl). The final concentrations of various factors and agents were as follows: 25 mM KCl in HK medium, 50 ng/ml IGF, 10 μM forskolin, and 10 mM lithium chloride.

For immunoprecipitation analysis, cells were placed on ice and harvested using non-denaturing lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na_3Vo_4, 1 μg/ml leupeptin). The lysates were centrifuged for 10 min at $10\,000\times g$ and the protein Akt was immunoprecipitated from 200 μg of cell-free extracts. The immune complexes were washed twice with lysis buffer. When in vitro kinase assay was carried out the immune complexes were washed with twice with kinase buffer (25 mM Tris (pH 7.5), 5 mM α-glycerophosphate, 2 mM DTT, 0.1 mM Na_3Vo_4, 10 mM $MgCl_2$). In vitro kinase assays were performed for 30 min at 30°C in 40 μl of reaction volume containing the 30 μl of immunoprecipitates in kinase buffer, 200 μM ATP. GSK-3 fusion protein (Cell Signaling Technology) was used as a substrate for Akt kinase activity. The reactions were terminated with 20 μl 3-x SDS sample buffer.

For Western blot analysis, cell lysates from granule neuron cultures and immunoprecipitates were resolved by 10% SDS–PAGE and transferred to polyvinyldipyrollidone fluoride (BioRad). The membrane was blocked in 5% non-fat dry milk for 30 min Tris-buffered saline containing tween-20. The membrane was incubated with an antibody overnight, diluted 1000-fold in 5% BSA solution. The secondary antibody was a HRP conjugated to the rabbit IgG or Sheep IgG diluted 4000-fold in the blocking buffer. The detection and quantification of protein was carried out using the ECL kit from Amersham–Pharmacia

Cultured cerebellar granule neurons undergo apoptosis when switched from HK medium to one with LK. Although neuronal death begins after about 16 h, commitment to death takes place within the first 6 h and characteristic features of apoptosis, such as chromatin condensation and DNA fragmentation can be observed after 8 h [4,23,39,48]. Apoptosis caused by K^+ deprivation can be inhibited by IGF-1, lithium and forskolin [15,16]. To examine whether the signaling pathways activated by these factors converge at Akt, we examined whether Akt is activated by these four factors. As shown in Fig. 1, treatment with all four factors resulted in Akt activation, consistent with the idea that it might represent a convergent point in survival promotion.

It has previously been shown that in granule neurons Akt is phosphorylated by IGF-1, although the sites of phosphorylation were not identified [20]. We have confirmed this finding using antibodies specific for Akt phosphorylated at Ser473 and Thr308. Both these antibodies are phospho-specific and do not recognize unphosphorylated Akt in our experience (not shown). As shown in Fig. 2 and consistent with growth factor-me-

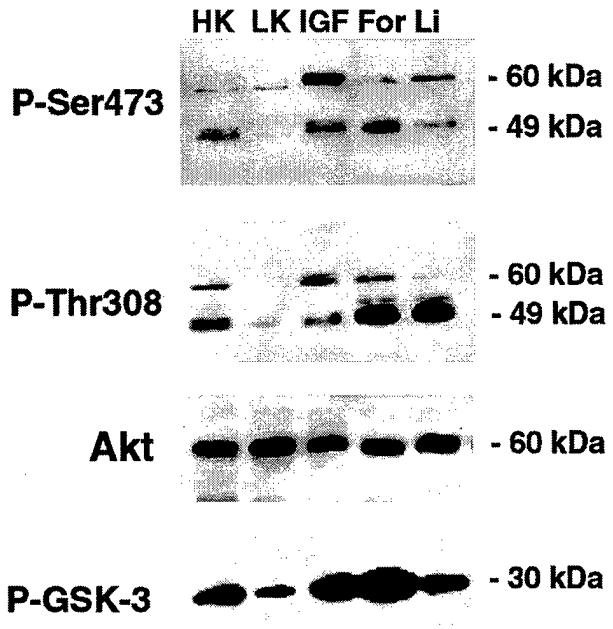

Fig. 1. Survival factors phosphorylate and activate Akt with distinct patterns. Seven days after plating, granule neuron cultures were switched to LK medium for 3 h to downregulate basal Akt activity. HK, IGF-1 (IGF, 25 ng/ml), forskolin (For, 10 μM), or lithium chloride (Li, 10 mM) was then added for 15 min after which the cells were lysed and Akt immunoprecipitated. The immunoprecipitate was used in in vitro kinase assays using Gsk-3 as substrate. The extent of Gsk-3 phosphorylation was examined by Western blotting using an antibody against phospho-Gsk-3 (bottom panel). The same blot was also analyzed using antibodies against phospho-Ser473, phospho-Thr308, or Akt (top three panels). The identity of the 49-kDa protein seen in the upper two panels is not known. The same results were obtained in three other independent experiments.

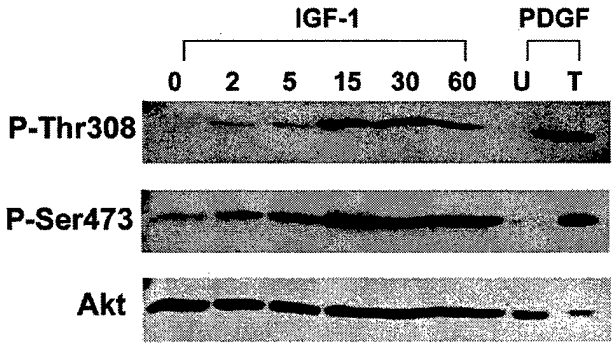

Fig. 2. Western blot analysis of Akt phosphorylation by IGF-1. IGF-1 phosphorylates Akt at Ser473 and Thr308. Cultures were switched from serum-containing HK medium to serum-free medium containing IGF-1 for 0–60 min and whole cell lysates prepared. As a control 3T3 cells were untreated (lane 'U') or treated (lane 'T') with PDGF (50 ng/ml) for 15 min. Immunoreactivity to phospho-Thr308 antibody (top panel), phospho-Ser473 antibody (middle panel) and Akt antibody (bottom panel) are shown. Robust phosphorylation can be seen at both sites, which is maximal at 15 min. In contrast to Thr308, a small amount of basal phosphorylation at Ser473 is evident.

diated Akt activation, treatment of granule neurons with IGF-1 results in increased phosphorylation at both Ser473 and Thr308. Increased phosphorylation at both sites is seen within 2 min and is maximal at 15 min, after which the level is somewhat reduced but persists for at least 60 min (Fig. 2). While Akt phosphorylation at Thr308 is not detectable in unstimulated cells, Ser473 is phosphorylated even before IGF-1 treatment (Fig. 2). That Ser473 is phosphorylated under unstimulated conditions has been confirmed using a monoclonal antibody that is totally specific for phosphorylated Ser473 and which does not cross-react with unphosphorylated Akt (not shown).

Treatment with HK also results in increased phosphorylation at Ther308 (Fig. 1). But in contrast to IGF-1, HK treatment does not increase phosphorylation of Ser473 beyond basal levels (Fig. 1), even when analysis was extended to 2 h following HK addition (unpublished observation). Basal levels of Ser473 phosphorylation does, however, appear to be necessary for neuronal survival as deprivation of all trophic support results in a reduction of phosphorylation to barely detectable levels within 6 h (Fig. 3), which coincides with the time at which these neurons are committed to death.

In contrast to both IGF-1 and HK, treatment with lithium treatment causes phosphorylation at Ser473 but not at Thr308. Finally forskolin treatment, which increases intracellular cAMP levels, results in increased phosphorylation at Thr308 but not at Ser473, a pattern that is different from that elicited by IGF-1 and lithium, but which is similar to HK treatment. Although results of Akt phosphorylation are shown at 15 min only in Fig. 1, the failure of lithium to phosphorylate Akt at Ther308, and of forskolin to phosphorylate Ser473, was confirmed in analysis extending for time-points ranging from 5 min to 2 h following addition of these agents.

Work done in a number of laboratories has shown that Akt phosphorylation at Thr308 is mediated by PDK-1 [1,2,28]. How Akt is phosphorylated at Ser473 is less clear but is believed to be a consequence of Thr308 phosphorylation. One hypothesis is that Ser473 may be phosphorylated by the integrin-linked kinase/PDK-2 [13]. More recent studies have found that Ser473 can also be phosphorylated by PDK-1 although this requires its interaction with a protein fragment, PIF [2]. It is also possible that Ser473 is phosphorylated by Akt itself following its phosphorylation at Thr308 [49]. Our finding that Ser473

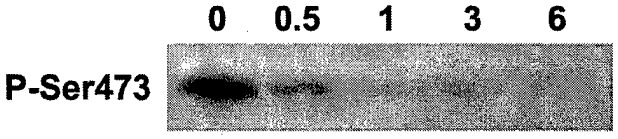

Fig. 3. Basal Ser473 phosphorylation decreases when switched to LK medium. Cultures were switched to serum-free medium for 0–6 h. Phospho-Ser473 immunoreactivity was examined at the various times following the switch.

phosphorylation is stimulated in the absence of Thr308 phosphorylation (following lithium treatment) argues against the Thr308-induced autophosphorylation model.

In addition to Akt, immunoprecipitation with Akt antibody pulls down a 49-kDa protein that is recognized by both the phospho-Ser473- and phospho-Thr308-specific antibody (Fig. 1). We find that the 49-kDa protein is also phosphorylated following treatment with the four survival factors albeit with a slightly different profile; it is most strongly phosphorylated by forskolin at a site that is recognized by the phospho-Ser473 and the phospho-Thr308 antibody, whereas IGF-1 has the least stimulatory effect. Three distinct isoforms of Akt have been identified and these are approximately 60-kDa in size [38,14,27]. Whether the 49-kDa protein represents another Akt isoform is not known at this time. One possibility is that this protein is serum-glucocorticoid kinase (SGK) a 49-kDa serine/threonine kinase that shares considerable structural similarity with Akt, and which like Akt, is rapidly phosphorylated by IGF-1 and serum [31,32,50]. While this manuscript was in preparation, Brunet et al. [6] demonstrated the ability of IGF-1 to stimulate SGK phosphorylation in granule neurons. Furthermore, these authors and another group [35] reported the ability of SGK to inhibit apoptosis in cell-lines. Previous work has shown that activation of SGK by IGF-1 requires phosphorylation at two sites — Thr256 and Ser422 [31]. The residues around Thr256 and Ser422 are virtually identical to that of Thr308 and Ser473 of Akt, respectively, making cross-reactivity of the phospho-Akt antibodies a possibility [31]. To test the possibility that the 49-kDa protein was SGK, immunoprecipitation was performed using Akt antibody and the immunoprecipitate subjected to immunoblotting using an SGK antibody. As shown in Fig. 4, the 49-kDa band is strongly recognized by the SGK antibody, which also cross-reacts to a small extent with Akt.

Previous studies have shown the ability of SGK to phosphorylate GSK-3 and other substrates commonly used to measure Akt activation, including the synthetic peptide Crosstide [31]. Thus, although our activity assays using immunoprecipitated Akt and Gsk-3 substrate are likely to primarily reflect Akt activation, given the fact that Akt and SGK share structural similarities and that the Akt antibody crossreacts with SGK, a contribution of SGK activity in assays cannot be excluded in our studies (and in other studies using the same approach).

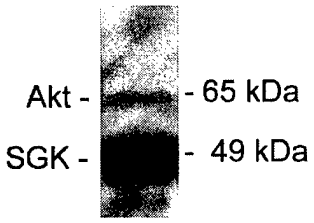

Fig. 4. Akt antibody recognizes SGK. Neuronal cultures were switched to serum-free medium for 3 h before treatment with HK. The cultures were lysed 15 min later and immunoprecipitated using an Akt antibody. The immunoprecipitate was subjected to Western blotting using an SGK antibody.

In summary — although HK, IGF-1, cAMP, and lithium phosphorylate Akt, they do so in three distinct patterns — while phosphorylation at both Ser473 and Thr308 is stimulated by IGF-1, HK and forskolin induce phosphorylation at Thr308 only, and lithium increases Akt phosphorylation at Ser473 alone. Therefore, contrary to what is generally believed, phosphorylation at the two Akt phosphorylation sites is not coordinated as commonly believed (for review, see Ref. [9]). Moreover, our results implicate SGK as another kinase that is activated by survival-promoting stimuli in neurons. Along with the two reports showing inhibition of apoptosis in cell-lines by SGK [6,35], our findings suggest that like Akt, SGK is a general inhibitor of apoptosis.

Acknowledgements

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Akt Is a Downstream Target of NF-κB*

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The ubiquitously expressed transcription factor NF-κB and the serine-threonine kinase Akt both are involved in the promotion of cell survival. Although initially believed to operate as components of distinct signaling pathways, several studies have demonstrated that the NF-κB and Akt signaling pathways can converge. Indeed, IκB kinase, the kinase involved in NF-κB activation, is a substrate of Akt, and activation of Akt therefore stimulates NF-κB activity. Although these results place Akt upstream of NF-κB activation in the sequence of signaling events, we report that this may not necessarily be the case and that Akt is a downstream target of NF-κB. Treatment of NIH3T3 cells with the NF-κB activators, tumor necrosis factor (TNF) α and lipopolysaccharide, results in the stimulation of Akt phosphorylation. The stimulation of Akt is, however, detected only after IκB-α degradation is induced by these agents. The nuclear translocation of p65 and increased DNA binding activity of NF-κB also precede Akt phosphorylation. Treatment with two pharmacological inhibitors of NF-κB, SN50 and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), blocks TNF-induced Akt activation. On the other hand TNF-mediated NF-κB activation is not reduced by the phosphoinositide-3 kinase inhibitors wortmannin and LY294002, although these inhibitors completely block the activation of Akt. These results suggest that NF-κB is required for TNF-mediated Akt activation and that it lies upstream of the stimulation of Akt. Consistent with this conclusion is the finding that overexpression of p65/RelA leads to Akt phosphorylation in the absence of extracellular stimulatory factors, whereas overexpression of IκB-α reduces Akt phosphorylation below basal levels. Interestingly, in addition to stimulating the phosphorylation of Akt, overexpression of p65 causes an increase in the expression of Akt mRNA and protein.

NF-κB plays a critical role in regulating inducible gene expression in immunity, stress responses, and inflammation (reviewed in Refs. 1–3). Moreover, NF-κB is involved in the regulation of cell survival (1–3). Although found to cause cell death in some cases, in a majority of systems NF-κB activation provides a survival-promoting signal (4). Classical NF-κB is a heterodimer composed of the p50 and p65/RelA subunits, which exists in the cytoplasm in an inactive complex bound by IκB proteins (1–3). Generally, the activation of NF-κB involves the phosphorylation of IκB, which then targets IκB for ubiquitination and degradation. This permits NF-κB to translocate to the nucleus, where it activates gene transcription. A wide variety of NF-κB-responsive genes have been identified. A key regulatory step in this pathway of NF-κB activation is the activation of a high molecular weight IκB kinase (IKK)[1] complex in which catalysis is thought to be performed by kinases including IKKα and IKKβ. Exactly how these IKKs are activated is the subject of much investigation. One well studied pathway that leads to NF-κB activation and which is activated by the cytokine TNFα involves the intracellular signaling molecules TNF receptor-associated factors (TRAF2 and TRAF6) and leads to the activation of NF-κB-inducing kinase (NIK), which phosphorylates the IKKs (1–3). More recent studies have shown that IKKα and IKKβ can be phosphorylated by an alternative pathway, which involves Akt. Indeed TNFα- and platelet-derived growth factor (PDGF)-induced NF-κB activation has been reported to require Akt (5–8), although this relationship is not always observed (9, 10). In addition to TNFα and PDGF, the activation of NF-κB by interleukin-1, bradykinin, interferon α/β, and bacterial proteins also has been reported to involve Akt activation (11–14).

Akt/PKB is a serine-threonine kinase that is best known for its ability to inhibit cell death pathways (reviewed in Refs. 15–17). It does so by directly phosphorylating and inactivating proteins involved in apoptosis including GSK3, Bad, Forkhead, and procaspase-9. Activation of Akt by growth factor and cytokine treatment generally occurs via the phosphoinositide 3-kinase (PI-3 kinase) pathway. Upon stimulation, PI-3 kinase phosphorylates specific phosphoinositide lipids, which accumulate in the plasma membrane, creating docking sites for Akt. At the plasma membrane Akt undergoes phosphorylation at two residues, Thr^{308} and Ser^{473}, leading to its activation.

Although the evidence for the involvement of Akt in NF-κB activation is compelling, whether NF-κB can affect Akt activity has not been examined. We report here that the expression and activity of Akt is regulated by NF-κB.

MATERIALS AND METHODS

Reagents and Plasmids—Recombinant human IGF-1 was purchased from Roche Molecular Biochemicals (Indianapolis, IN), recombinant human TNFα was purchased from Promega (Madison, WI), and (lipopolysaccharide) LPS was purchased from Sigma. Actinomycin D, cycloheximide, SN-50, wortmannin, and LY294002 were purchased from Calbiochem (La Jolla, CA), and TPCK was purchased from Sigma. The plasmids encoding p65-FLAG (CMV-p65) and IκB-α (CMV-IκBα) were gift from A. S. Baldwin (University of North Carolina) and R. Gaynor (University of Texas Southwestern Medical Center), respectively. The CMV-Akt-Myc was purchased from Upstate Biotechnology (Lake Placid, NY).

Rabbit polyclonal antibodies against IκB-α and p65 and the mouse monoclonal Myc antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against total Akt,

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[1] The abbreviations used are: IKK, IκB kinase; IGF-1, insulin-like growth factor-1; LPS, lipopolysaccharide; PI-3 kinase, phosphoinositide-3 kinase; RT-PCR, reverse transcriptase-PCR; TNF, tumor necrosis factor; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TRAF, TNF receptor-associated factor; NIK, NF-κB-inducing kinase; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay.

Ser(P)^{473} Akt, Akt2, Akt3, and phospho-GSK3 were from Cell Signaling, Inc. (Beverly, MA). Polyclonal β-tubulin antibody was purchased from Sigma. Peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology.

Cell Lines and Neuronal Cultures—NIH3T3 cells and HEK293 cell lines were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml), which were all obtained from Invitrogen.

Cerebellar granule neurons were cultured from dissociated cerebella of 7–8-day-old rats as described previously (18). Cells were plated in basal Eagle's medium with Earle's salts (Invitrogen) supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine (Invitrogen), and 100 μg/ml gentamycin on dishes coated with poly-L-lysine at a density of 1.0×10^6 cells/well, 1.2×10^7 cells/60-mm dish, or 3.0×10^7 cells/100-mm dish. Cytosine arabinofuranoside (10 μM) was added to the culture medium 18–22 h after plating to prevent replication of non-neuronal cells. The cultures were maintained for 6–7 days prior to experimental treatments.

Treatment with Stimulators and Inhibitors—When NIH3T3 cells were treated with TNFα or LPS, cultures at about 80% confluency on 60-mm dishes were used. Prior to treatment, the cells were maintained in Dulbecco's modified Eagle's medium containing 0.5% fetal calf serum for 24 h. When used, SN-50 (10 μM) and TPCK (10 μM) were added to the medium 1 h prior to the addition of TNFα or LPS whereas wortmannin and LY294002 were added 2 h prior to the addition. Pretreatment with actinomycin D and cycloheximide was performed for 1 h.

For treatment with IGF-1, cerebellar granule neuron cultures were switched to serum-free Basal Eagle's medium with Earle's salts for 3 h prior to the addition of IGF-1 (25 ng/ml). When used, SN-50 and TPCK were added 2 h prior to stimulation with IGF-1.

Transient Transfections—NIH3T3 cells and HEK293-T cells were plated at a density of 6×10^5 per 60-mm dish in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were carried out the following day using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's guidelines. 5–6 μg of plasmid DNA was used per dish, and the transfections were carried out for 4–6 h in Opti-MEM (Invitrogen) medium. The transfection medium was then replaced for 48 h by serum-containing Dulbecco's modified Eagle's medium (unless specified otherwise).

Western Blots—After treatment, confluent cell monolayers in 60-mm dishes were washed twice with ice-cold phosphate-buffered saline and lysed by incubation for 20 min in 1 ml of ice-cold cell lysis buffer (1% Nonidet P-40, 50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 1 mM sodium fluoride, and 1× protease mixture) and stored at −70 °C. Protein concentrations were measured using a Bradford protein assay kit (Bio-Rad). Equivalent amounts of protein were resolved and mixed with 6× SDS-PAGE sample buffer and then subjected to SDS-PAGE in a 4–20% linear gradient Tris-HCl-ready gel (Bio-Rad, Hercules, CA). The resolved proteins were transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% nonfat dry milk (except for phospho-Akt Western blots in which 5% bovine serum albumin was used) in Tris-buffered saline, pH 7.4, containing 0.05% Tween 20 and were incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies according to the manufacturer's instructions. The protein of interest was visualized with enhanced chemiluminescent (ECL, Amersham Biosciences) reagents. Images of the bands were scanned by reflectance scanning densitometry, and the intensity of the bands was quantified using NIH Image software.

Akt Activity Assays—Akt assays were performed on Akt that was immunoprecipitated from neuronal cultures or cell lines using a kit from Cell Signaling Technologies. Following treatment, the cultures were placed on ice and harvested using non-denaturing lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, and protease inhibitors). The lysates were centrifuged for 10 min at 10,000 × *g*, and the protein Akt was immunoprecipitated from 200 μl of cell-free extracts. The immune complexes were washed twice with lysis buffer. When *in vitro* kinase assay was carried out the immune complexes were washed twice with kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium vanadate, and 10 mM $MgCl_2$). *In vitro* kinase assays were performed for 30 min at 30 °C in 40 μl of reaction volume containing the 30 μl of immunoprecipitates in kinase buffer, 200 μM ATP. GSK3 fusion protein (Cell Signaling Technology) was used as a substrate for Akt kinase activity. The reactions were terminated with 20 μl of 3× SDS sample buffer and subjected to Western blotting using a phospho-GSK antibody (Cell Signaling Technology).

Preparation of Nuclear and Cytoplasmic Extracts—To obtain cytoplasmic proteins, cells were washed with cold phosphate-buffered saline (pH 7.2), resuspended in buffer containing 10 mM HEPES pH 7.6, 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, 50 mM sodium fluoride, 50 mM β-glycophosphate, 5% glycerol, and 1× protease inhibitor mixture (Roche Molecular Biochemicals), and incubated on ice for 15 min. At the end of incubation, 0.05 volumes of 10% Nonidet P-40 was added. Cells were vortexed for 30 s and then subjected to micro-centrifugation for 30 s. Supernatants were collected as cytoplasmic extracts. The protein concentrations of the cytoplasmic extracts were determined using Bio-Rad reagent.

Nuclei from NIH3T3 cells were resuspended in buffer containing 20 mM HEPES pH 7.6, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 1× protease inhibitor mixture and extracted on ice for 30 min followed by micro-centrifugation at 14,000 rpm for 10 min. The supernatants were collected as nuclear extracts. Concentrations of these nuclear extracts were determined by the Bradford method using reagents from Bio-Rad.

Gel Electrophoresis Mobility Shift Assay—10 μg of each nuclear extract sample was incubated with 0.1 pmol of ^{32}P-labeled double-stranded κB binding oligonucleotide (5′-GCTGGGGACTTTC-3′) or SP1 binding oligonucleotide (5′-ATTCGATCGGGGCGGGGCGAGC-3′) in buffer containing 1 μg of poly(dI·dC), 1 μg of bovine serum albumin, 10 mM HEPES pH 7.6, 0.5 mM dithiothreitol, 0.1 mM EDTA, 60 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM $MgCl_2$, and 12% glycerol at room temperature for 30 min. Samples were analyzed by 5% native PAGE followed by autoradiography. For competition and antibody-mediated supershift experiments, antibodies or oligonucleotides were added to the reaction for 15 min at room temperature before the addition of radiolabeled oligonucleotide probe.

Isolation of RNA and Semi-quantitative RT-PCR—The mRNA levels of Akt in different plasmid-transfected cells were analyzed by semi-quantitative RT-PCR. 1 μg of total RNA, isolated using an RNA isolation kit (Invitrogen), was used in reverse transcription reactions as described by the manufacturer. The resulting total cDNA was then used in the PCR to measure the mRNA levels of Akt. The mRNA level of β-actin was used as internal control. PCR was carried out with *Taq* polymerase, and conditions were as follows: pre-denaturing at 94 °C for 3 min and 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. The sequences of the primers used were upstream primer (5′-ATCGTCGCCAAGGATGAGGT-3′) and downstream primer (5′-TCTCGTGGTCCTGGTTGTAG-3′) for Akt and upstream primer (5′-TCACCAACTGGGACGACATG-3′) and downstream primer (5′-AGTCCTGTGGCATCCACGAA-3′) for β-actin.

RESULTS

TNFα is a commonly used activator of NF-κB. Treatment with TNFα causes the rapid degradation of IκB proteins, permitting the nuclear translocation of NF-κB and resulting in increased DNA binding activity of NF-κB (1–3). As shown in Fig. 1*A*, besides stimulating IκB breakdown, treatment of NIH3T3 cells with TNFα increases Akt phosphorylation. Stimulation of Akt by TNFα has been observed by other laboratories (5–8) and has led to the conclusion that Akt can stimulate NF-κB activity. As shown in Fig. 1, *A* and *B*, however, following TNFα treatment the reduction in IκB-α levels is detectable after 5 min, whereas the increase in Akt phosphorylation occurs only after 15 min. As shown in Fig. 2, the translocation of p65/RelA to the nucleus and the resulting increase in DNA binding activity of p65 can be observed within 10 min of TNFα-treatment, which is also before the phosphorylation of Akt can be detected.

Another agent that is commonly used to activate NF-κB *in vivo* and in culture is LPS. As shown in Fig. 1*C*, LPS treatment also stimulates Akt phosphorylation. The increase in Akt phosphorylation occurs at 1 h whereas the decrease in IκB-α levels is detectable after 30 min (Fig. 1, *C* and *D*). Taken together, these results show that the stimulation of Akt phosphorylation by TNFα and LPS begins after NF-κB activation has occurred,

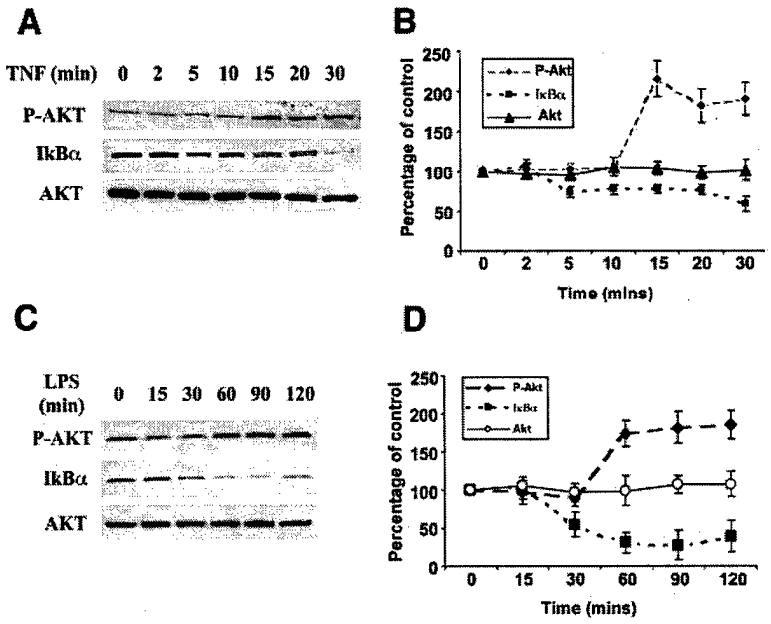

FIG. 1. **TNFα and LPS treatment stimulates IκB-α degradation and Akt phosphorylation.** NIH3T3 cells were treated with 10 ng/ml TNFα (*A* and *B*) or 100 nM LPS (*C* and *D*) for various time periods as indicated above the *panels*. Following treatment whole cell lysates were prepared, and the lysates were analyzed by Western blot using an IκB-α antibody. The same blot was stripped and reprobed with antibodies against Ser(P)^{473} Akt (*P-AKT*) and total Akt (*AKT*). *A* and *C*, protein levels after treatment with TNFα and LPS, respectively. Densitometric analysis of the blots from three separate experiments was performed, and the relative levels of IκB-α and phospho-Akt after TNFα and LPS treatments are shown in *B* and *D*, respectively. With TNFα treatment (*A* and *B*) reduced IκB-α occurred at 5 min whereas stimulation of Akt phosphorylation was seen at 15 min. With LPS treatment (*C* and *D*), IκB-α degradation occurred at 30 min whereas increased Akt phosphorylation was detected at 1 h.

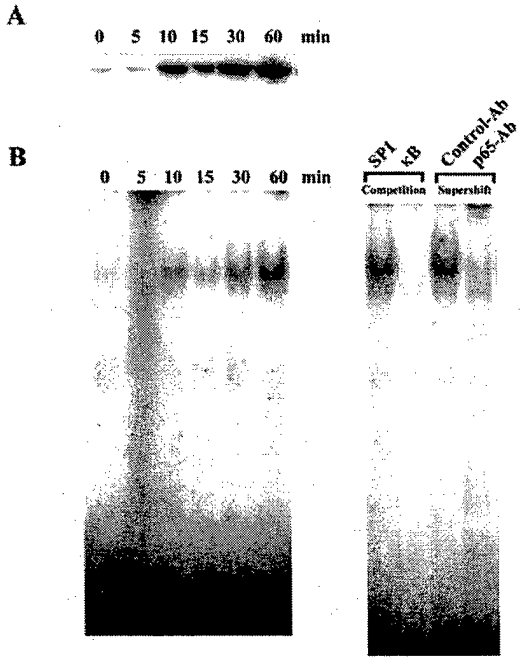

FIG. 2. **Nuclear translocation and DNA binding activity of p65 following TNFα treatment.** NIH3T3 cells were treated with 10 ng/ml TNFα for various time periods as indicated above the *panels*. Following treatment the cells were lysed, and nuclear extracts were prepared. An aliquot of the extracts was used for Western blotting while the rest was used in EMSA assays. *A*, results of Western blot analysis using an antibody against p65. *B*, results of EMSA using a radiolabeled oligonucleotide containing the consensus NF-κB binding site as probe. *Lanes* marked *κB* and *SP1* are control lanes that demonstrate specificity of binding in which a 100-fold excess of unlabeled NF-κB oligonucleotide or a nucleotide containing the SP1 binding site was included in the binding reaction. In the *lanes* marked *p65-Ab* and *Control-Ab*, a preincubation of the extract with p65 antibody or preimmune serum was performed. NF-κB binding activity was disrupted by p65 antibody. Similar supershift assays using antibodies against p50, c-rel, and relB failed to affect binding activity appreciably (data not shown).

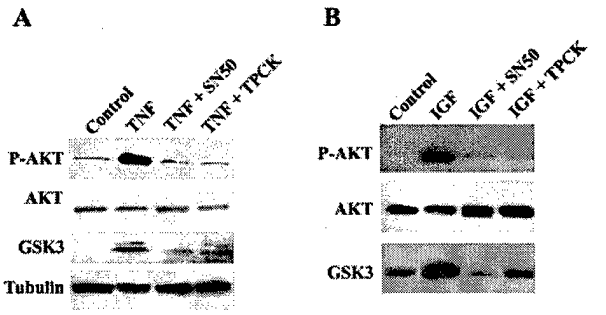

FIG. 3. **Pharmacological inhibitors of NF-κB inhibit phosphorylation of Akt by TNFα and IGF-1.** NIH3T3 cells and primary cerebellar granule neurons were treated with TNFα (10 ng/ml) and IGF-1 (25 ng/ml), respectively. The effect of SN-50 (10 μM) and TPCK (10 μM) on the stimulation of Akt phosphorylation was examined by Western blot using an antibody against Ser(P)^{473} Akt. The same blot was reprobed with antibodies against Akt and β-tubulin. Control cells received medium with no additives. *A*, NIH3T3 cells were treated for 30 min with no additives (*control*) or TNFα alone or were co-treated with TNFα and SN-50 or TNFα and TPCK. Whole cell lysates were prepared, and a portion of the lysates was analyzed for Akt phosphorylation. The rest of the lysate was used for assaying Akt activity. Here Akt was immunoprecipitated from the lysates and included in an *in vitro* kinase assay using a synthetic GSK3 peptide as a substrate. Phosphorylation of GSK3 was detected using a phospho-GSK-specific antibody by Western blot analysis of the lysates. *B*, cerebellar granule neurons were switched to serum-free medium for 3 h and then treated with no additives (*control*) or IGF-1 or were co-treated with IGF-1 and SN-50 or IGF-1 and TPCK. Following lysis of the neuronal cultures, Akt was immunoprecipitated and used in *in vitro* kinase assays with GSK3 peptide as substrate. Following PAGE, the phosphorylation of GSK3 was detected by Western blot using a phospho-GSK3 antibody. The same blot was reprobed with antibodies against phospho-Akt and total Akt. In the presence of SN-50 and TPCK the level of Akt phosphorylation was reduced to that seen in the control cultures.

raising the possibility that NF-κB may be involved in stimulating Akt phosphorylation.

To examine whether the stimulation of Akt is in fact a consequence of NF-κB activation, we used SN-50, a cell-permeable peptide that inhibits NF-κB by blocking its translocation to the nucleus (19). Previous studies have demonstrated that SN-50 is highly selective against NF-κB and has no effect on the activities of any other signaling molecule (20). Treatment with SN-50 reduced Akt phosphorylation and activation by TNFα (Fig. 3*A*). As shown in Fig. 3*A*, a similar reduction of Akt phosphorylation and activity is seen with TPCK, another commonly used pharmacological inhibitor of NF-κB that acts by inhibiting IκB degradation (21–23). To examine whether the inhibitory effect of SN-50 and TPCK occurred in other cell

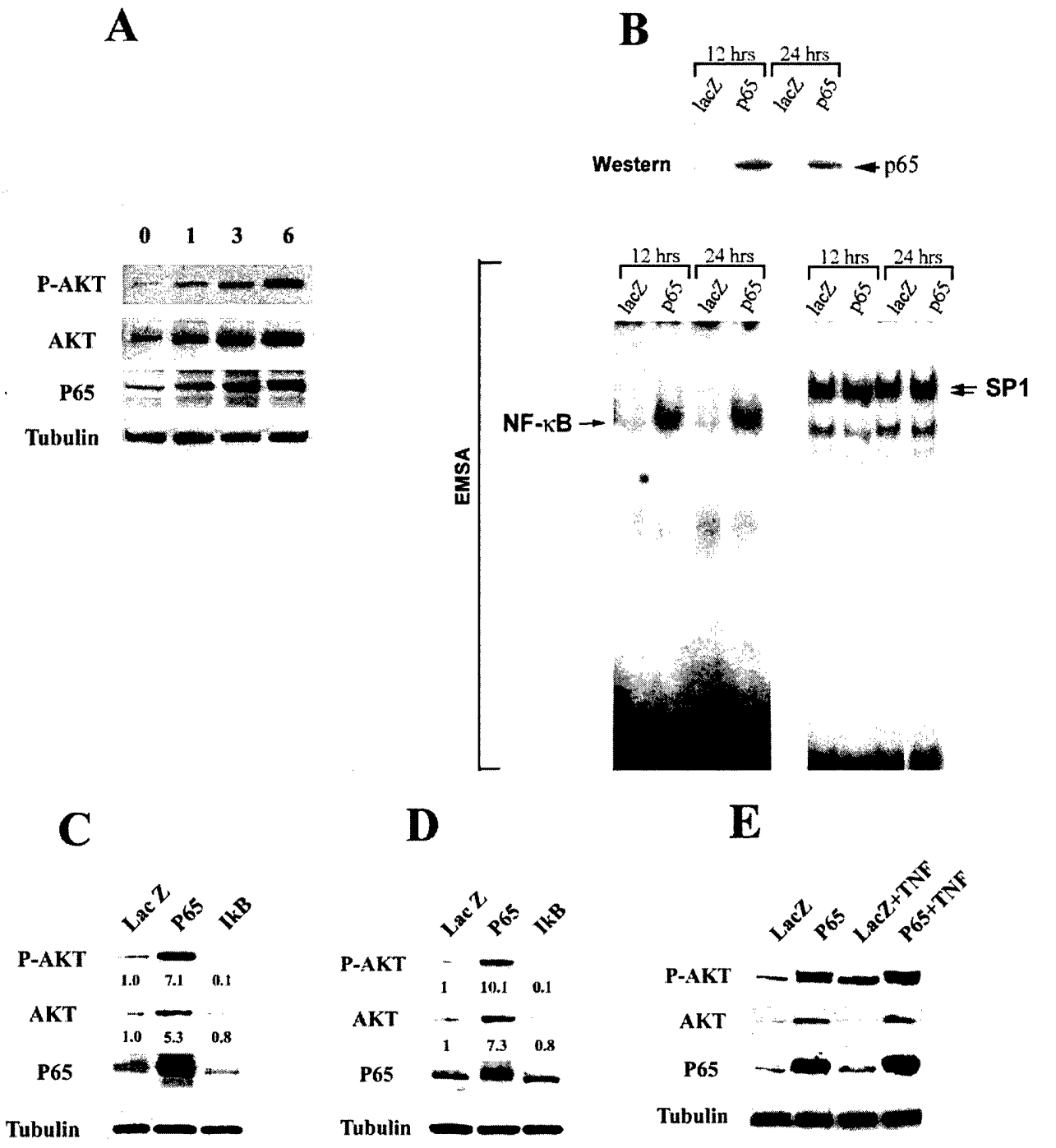

FIG. 4. **Overexpression of p65 stimulates Akt phosphorylation in the absence of TNFα.** *A*, NIH3T3 cells were transfected with 0, 1, 3, and 6 μg of CMV-p65 plasmid DNA. Whole cell lysates were prepared 24 h after transfection and analyzed for Akt phosphorylation using a Ser(P)^{473} Akt antibody. The blots were reprobed with antibodies against total Akt, p65, and β-tubulin. The extent of Akt phosphorylation increases proportionally to the level of p65. *B*, NIH3T3 cells were transiently transfected with CMV-lacZ or CMV-p65-FLAG. Nuclear extracts were prepared 12 and 24 h later and used in Western blot analysis using a p65 antibody (*upper panel*) and in EMSA assays (*lower panels*) using oligonucleotide probes containing the consensus sequences for NF-κB (*left*) or SP1 binding (*right*). The binding activity of NF-κB but not of SP1 is increased with p65 overexpression. *C* and *D*, NIH3T3 (*C*) and HEK293 (*D*) were transiently transfected with CMV-lacZ, CMV-p65-FLAG, or CMV-IκBα. Whole cell lysates were prepared 24 h after transfection and analyzed for Akt phosphorylation using a Ser(P)^{473} Akt antibody. The blots were reprobed with antibodies against total Akt, p65, and β-tubulin. The -fold change in phospho-Akt and Akt immunoreactivity following p65 and IκB-α expression relative to control cells (expressing lacZ) determined by densitometric analysis is shown below the *panels*. *E*, the effect of p65 overexpression on the TNFα-mediated stimulation of Akt phosphorylation at Ser^{473} was examined. Twenty four hours after transfection with lacZ- or p65-expressing plasmids, the cultures were treated with TNF (10 ng/ml) for 30 min before lysis of the cells. Addition of TNFα does not increase the extent of Akt phosphorylation over that seen with p65 overexpression alone.

types and by stimuli other than TNFα, we used primary cultures of cerebellar granule neurons. Treatment of cerebellar neurons with IGF-1 causes Akt phosphorylation and activation (24, 25). As shown in Fig. 3*B*, pretreatment with SN-50 or TPCK substantially reduces Akt phosphorylation induced by IGF-1 treatment, consistent with the involvement of NF-κB in

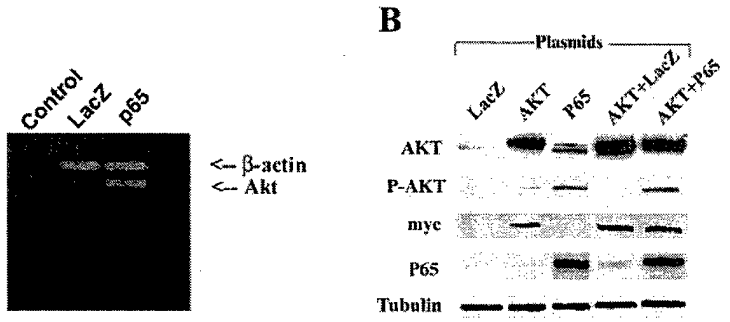

FIG. 5. **p65-mediated increase of Akt expression is transcriptionally mediated.** *A*, analysis of Akt mRNA. NIH3T3 cells were transfected with CMV-lacZ or CMV-p65. Twenty fours hours after transfection the cultures were lysed, and total RNA was prepared. Equal amounts of RNA (1 μg) were used in RT-PCR reactions using primers specific for Akt and β-actin. The *lane* marked *control* shows the result of amplification in the absence of RNA. A robust elevation of Akt mRNA expression is seen in cultures overexpressing p65. In contrast, p65 had no effect on β-actin mRNA. RT-PCR analysis was performed using two other primer pairs that amplified different regions of Akt mRNA. Although the data is not shown, a similar increase of Akt mRNA was observed. *B*, NIH3T3 cells were transfected with the following plasmids: CMV-lacZ (*LacZ*), CMV-Akt-Myc (−Myc *AKT*), CMV-p65 (*P65*), CMV-lacZ + CMV-Akt-Myc (*LacZ* + *AKT*), or CMV-p65 + CMV-Akt-Myc (*P65* + *AKT*). The expression and phosphorylation of endogenous Akt is increased by p65. Expression of Akt driven off the CMV promoter was detected using an antibody against the Myc epitope tag. No change was seen in the levels of transfected Akt as judged by the similar intensity of the Myc immunoreactive bands. The same blot was reprobed with antibodies against p65 (to confirm expression of transfected p65) and β-tubulin (to control for equal loading of proteins).

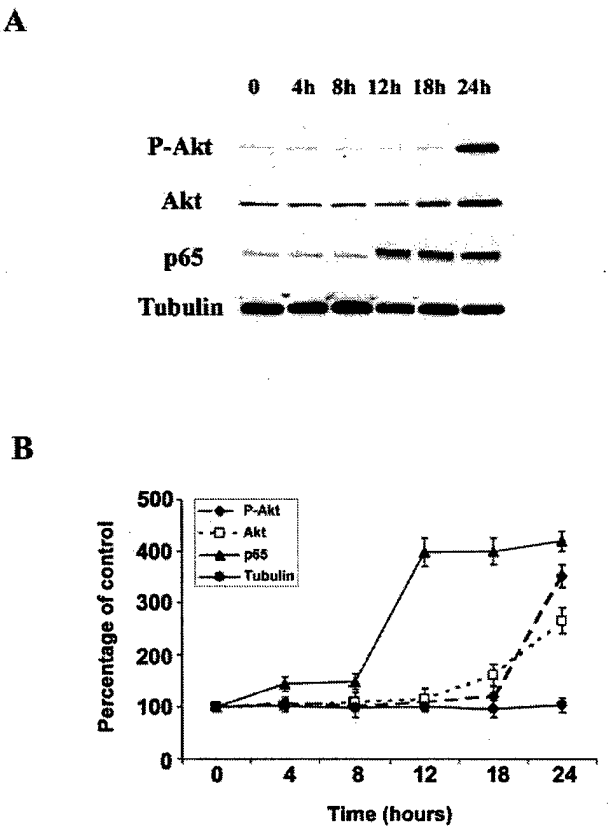

FIG. 6. **Time course of p65-mediated induction of Akt expression and phosphorylation.** NIH3T3 cells were transfected with CMV-p65. Lysates were prepared at various time points ranging from 0–24 h after transfection as indicated above the *lanes*. The lysates were subjected to Western blot analysis using antibodies against Ser(P)^{473} Akt and total Akt. The same blot was reprobed with an antibody against p65 and β-tubulin. Whereas *A* shows the results of a representative experiment, *B* shows the data obtained from three independent experiments. Although high p65 expression is seen within 12 h, the increase in Akt expression is clearly evident only at 18 h after transfection. Stimulation of Akt phosphorylation is seen at 24 h.

the stimulation of Akt phosphorylation.

Although both SN-50 and TPCK are commonly used NF-κB inhibitors, the possibility that these compounds may affect Akt by an NF-κB-independent mechanism cannot be completely excluded. To more directly determine whether NF-κB affects Akt, we transfected NIH3T3 cells with p65/RelA and examined its effect on Akt phosphorylation. Transfection using a p65 expression vector resulted in a dose-dependent increase in total p65 levels (Fig. 4*A*). The increased p65 levels resulted in a robust increase in NF-κB binding activity but not in the binding activity of another transcription factor, SP1 (Fig. 4*B*). As shown in Fig. 4, *A* and *C* and consistent with the idea that Akt is a downstream target of NF-κB, overexpression of p65 caused an increase in Akt phosphorylation. Overexpression of IκB-α on the other hand, which would lead to inhibition of NF-κB translocation, showed reduced Akt phosphorylation compared with control cells that overexpressed lacZ (Fig. 4*C*). To rule out the possibility that the stimulatory effect of p65 on Akt was restricted to NIH3T3 cells, we performed a similar experiment using the HEK293 cell line. As shown in Fig. 4*D*, stimulation of Akt phosphorylation by p65 was seen in HEK293 cells also. When TNFα was applied to cells overexpressing p65, the induction of Akt phosphorylation was not higher than that seen with p65 alone, suggesting that similar mechanisms were involved in the stimulatory effects of p65 and TNFα (Fig. 4*E*).

Interestingly, in addition to inducing Akt phosphorylation, overexpression of p65 caused an increase in the level of total Akt expression in both NIH3T3 and HEK293 cells (Fig. 4, *A*, *C*, and *D*). The overexpression of IκB-α reduced Akt expression to a level slightly below that seen in lacZ-expressing cells (Fig. 4, *C* and *D*). In addition to increasing the level of Akt protein, overexpression of p65 caused an increase in Akt mRNA, ruling out post-translational mechanisms as responsible for the stimulatory effect of p65 (Fig. 5*A*). As a step toward determining whether the increase in Akt expression occurred at the transcriptional level as opposed to increasing mRNA stability, we expressed Akt using a CMV-driven promoter. As shown in Fig. 5*B*, although the expression of endogenously produced Akt is increased by p65 overexpression, the level of Akt produced via a CMV promoter is unchanged by p65, arguing against increased mRNA as the mechanism by which Akt expression is stimulated.

To examine the time course of Akt induction following p65 expression, we transfected NIH3T3 cells with CMV-p65 and examined the effect on Akt expression and phosphorylation. As shown in Fig. 6, *A* and *B*, although the increase in p65 expression is seen at 12 h, the stimulation of Akt expression occurs at

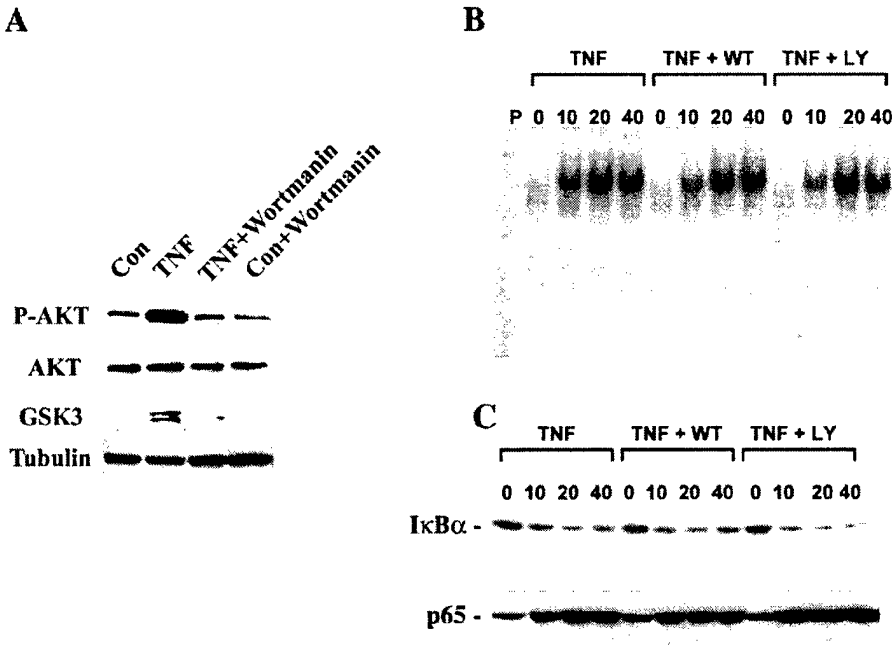

FIG. 7. **Inhibition of PI-3 kinase blocks Akt activation by TNF but has no effect on NF-κB activation.** *A*, effect of PI-3 kinase inhibition on Akt phosphorylation and activity. Control cultures (*Con*, receiving no additives) or TNF-treated cells were co-treated with or without 1 μM wortmannin for 30 min. Whole cell lysates were prepared and subjected to Western blotting using antibodies against Ser(P)^{473} Akt, total Akt, and β-tubulin. In addition, Akt was immunoprecipitated from the lysates and used in *in vitro* kinase assays with GSK3 substrate. Phosphorylation of GSK3 by the immunoprecipitated Akt was analyzed by Western blotting using a phospho-GSK3 antibody. Similarly LY294002 (100 μM) and lower doses of wortmannin (100–500 nM) were also found to fully block Akt phosphorylation (not shown). *B* and *C*, effect of PI-3 kinase inhibition on p65 translocation, IκB-α degradation, and NF-κB activity. Cultures were treated with TNFα alone or co-treated with TNFα and 500 nm wortmannin (*TNF + WT*) or TNFα and 50 μM LY294002 (*TNF + LY*) for 0, 10, 20, and 40 min. Nuclear extracts and cytoplasmic extracts were prepared. *B*, nuclear extracts were used for EMSA with a radiolabeled NF-κB oligonucleotide probe; *lane P*, probe only. *C*, aliquots of nuclear and cytoplasmic extracts from the same experiment were used in Western blots with p65 and IκB-α antibodies, respectively.

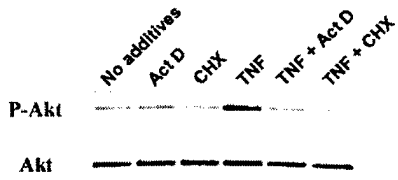

FIG. 8. **Inhibitors of gene expression block TNF-induced Akt phosphorylation.** NIH3T3 cells were treated with 10 μg/ml actinomycin D (*Act D*) or 50 μg/ml cycloheximide (*CHX*) in the absence or presence of 10 ng/ml TNFα. Whole cell lysates were prepared and analyzed by Western blotting using antibodies against phospho-Akt and total Akt. A 1-h pretreatment with the inhibitors was included before addition of TNFα.

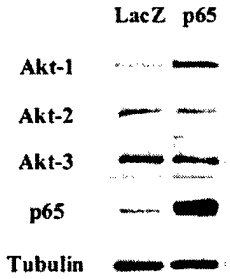

FIG. 9. **Akt2 and Akt3 expression is not affected by p65 overexpression.** Whole cell lysates from NIH3T3 cells transfected for 24 h with CMV-p65-FLAG were analyzed by Western blotting using antibodies specific for Akt, Akt2, or Akt3. The blot was also analyzed using p65 and tubulin antibodies.

18 h. The increased phosphorylation of Akt follows the increased level of expression and is detectable at 24 h.

Following treatment with a variety of growth factors and cytokines, the phosphorylation of Akt is mediated by a PI-3 kinase-dependent mechanism (15–17). Not surprisingly, therefore, treatment with the PI-3 kinase inhibitor wortmannin inhibits TNF-induced Akt phosphorylation and activity (Fig. 7*A*). However, the increased DNA binding activity of NF-κB following TNFα treatment is not substantially reduced by wortmannin or by LY294002, two specific inhibitors of PI-3 kinase, even when used at relatively high concentrations (Fig. 7*B*). Similarly, translocation of p65 to the nucleus and reduction of IκB-α levels in the cytoplasm is not affected by wortmannin (Fig. 7*C*). This observation is consistent with Akt being downstream of NF-κB in the sequence of events activated by TNFα, and it places PI-3 kinase in between NF-κB and Akt. To gain insight into the mechanism by which TNFα-mediated NF-κB activation stimulates Akt phosphorylation, we examined the effect of the transcriptional inhibitor actinomycin D and the translational inhibitor cycloheximide. As shown in Fig. 8, both actinomycin D and cycloheximide prevented the increase in Akt phosphorylation by TNFα, suggesting the need for new gene expression.

Akt generally refers to Akt1, one of three known members of the Akt gene family (15–17). Although it is likely that the three Akt proteins have at least some non-overlapping functions, these have yet to be fully understood. It is known that mice lacking Akt1 have defects in the induction of apoptosis and are growth-retarded (27), whereas Akt2-lacking mice display defects in the capacity of insulin to reduce blood glucose (32). To examine whether p65 also increases expression of Akt2 and Akt3, we used antibodies specific for these two forms in Western blot analysis. In contrast to its effect on Akt, p65 had no discernible effect on the expression of Akt2 and Akt3 (Fig. 9).

DISCUSSION

Our results indicate that NF-κB is involved in the stimulation of Akt activity. NF-κB-activating stimuli such as TNFα and LPS also stimulate Akt phosphorylation and activity. Events necessary for the activation of NF-κB (such as IκB degradation, nuclear translocation, and increase in NF-κB DNA binding) all occur before the increase in phosphorylation of Akt is detectable. TNFα-mediated phosphorylation and activation of Akt is blocked by two independent pharmacological inhibitors of NF-κB. Finally, overexpression of p65 leads to higher Akt phosphorylation, whereas similar overexpression of IκB-α inhibits it. The stimulatory effect of NF-κB on Akt is not restricted to cell lines and is seen in primary cultures of cerebellar granule neurons. The mechanism by which NF-κB activates Akt phosphorylation is unclear. Our finding that the stimulation of Akt by TNFα can be blocked by inhibitors of gene transcription and translation suggest the requirement for the synthesis of new proteins, an event likely regulated by the activation of NF-κB.

Several recent reports have described that NF-κB activation by TNFα is mediated by Akt. Contrary to the findings of Ozes *et al.* (5) and consistent with the findings of other groups (6, 13, 26, 28), we find that wortmannin, even when used at high doses, does not affect TNF-mediated translocation of NF-κB to the nucleus or its DNA binding activity. It does, however, inhibit Akt phosphorylation by TNFα efficiently, as reported previously (5–7, 13, 28). Assuming that the three molecules are part of a common TNF-activated signaling cascade, our results place PI-3 kinase upstream of Akt but downstream of NF-κB. Other studies have shown that the expression of dominant-negative Akt fails to reduce NF-κB translocation or DNA binding activity (7, 13) and that Akt overexpression by itself is unable to activate NF-κB translocation in the absence of TNFα (7). In contrast, we find that overexpression of p65 in the absence of any extracellular signals is capable of robustly stimulating Akt phosphorylation. While not ruling out the possibility that Akt activates NF-κB as reported previously (5–7), our results indicate that Akt can be a downstream target of NF-κB.

Interestingly, in addition to stimulating its phosphorylation and activity, overexpression of p65 increases the expression of Akt mRNA and protein. The fact that the level of Akt produced from a CMV promoter is not increased by p65 overexpression suggests that the induction of Akt expression is caused by increased transcription rather than increased mRNA stability. It is not known at this time whether this is a direct effect of NF-κB on the Akt promoter. The delayed activation of Akt following p65 overexpression raises the possibility that other intermediary proteins are involved. In contrast to the overexpression of 65, which induces both expression and phosphorylation of Akt, treatment with TNFα stimulates Akt phosphorylation only. The reason for this difference presently is not known, but it may be related to the level of p65 expression. In contrast to transient transfection, which produces large quantities of p65, TNFα treatment results in a relatively modest translocation of p65. Moreover, in contrast to the sustained production of p65 by transfection, TNF-mediated stimulation of NF-κB is transient. The physiological relevance of increased Akt production in response to sustained p65 overexpression is therefore unclear. It is noteworthy, however, that sustained up-regulation of NF-κB has been observed to occur in some cancers as discussed below. It would be of interest to examine whether in such cancers the expression and activity of Akt are also elevated.

NF-κB can directly activate proto-oncogenes such as cyclinD1 and c-Myc (29–31) and has thus been implicated in the development or progression of human cancers. Moreover, chromosomal amplification, overexpression, and rearrangement of genes coding for NF-κB proteins have been found to occur in many human hematopoietic and solid tumors, and the high constitutive activity of NF-κB noted in Hodgkin's lymphoma has been attributed to a mutation in the IκB-α gene (reviewed in Ref. 31). In addition to providing a direct stimulus toward proliferation, NF-κB can contribute to the development of cancer by suppressing apoptosis, and several anti-apoptotic genes whose expression is activated by NF-κB have been identified, including those encoding cellular inhibitors of apoptosis proteins, manganese superoxide dismutase, A20, and the anti-apoptotic Bcl-2 proteins Bfl-1/A1 and Bcl-XL (reviewed in Ref. 4). We now show that another downstream target of NF-κB is Akt. Given the established role of Akt in the inhibition of apoptosis and its direct involvement in certain cancers such as ovarian cancer, prostate cancer, and gastric adenocarcinomas (reviewed in Ref. 17), the activation of Akt by NF-κB may serve as a powerful stimulus in the development or progression of cancer.

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Apoptosis in cerebellar granule neurons is associated with reduced interaction between CBP and NF-κB

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Abbreviated title: NF-κB and CBP in neuronal apoptosis

Abstract

Cerebellar granule neurons undergo apoptosis when switched from medium containing depolarizing levels of potassium (high K+ medium, HK) to medium containing low K+ (LK). NF-κB, a ubiquitously expressed transcription factor, is involved in the survival-promoting effects of HK. However, neither the expression or the intracellular localization of the five NF-κB proteins, or of IκB-α and IκB-β are altered in neurons primed to undergo apoptosis by LK suggesting that uncommon mechanisms regulate NF-κB activity in granule neurons. In this study, we show that p65 interacts with the transcriptional coactivator, CREB-binding protein (CBP), in healthy neurons. The decrease in NF-κB transcriptional activity induced by LK treatment is accompanied by a reduction in the interaction between p65 and CBP an alteration that is accompanied by hyperphosporylation of CBP. LK-induced CBP hyperphosphorylation can be mimicked by inhibitors of protein phosphatase 2A (PP2A) and PP2A-like phosphatases such as okadaic acid and cantharidin, which also causes a reduction in p65-CBP association. In addition, treatment with these inhibitors induces cell-death. Treatment with high concentrations of the broad-spectrum kinase inhibitor staurosporine prevents LK-mediated CBP hyperphosphorylation and inhibits cell death. In vitro kinase assays using GST-CBP fusion proteins map the LK-regulated site of phosphorylation to a region spanning residues 1662 - 1840 of CBP. Our results are consistent with possibility that LK-induced apoptosis is triggered by CBP hyperphosphorylation, an alteration that causes the dissociation of CBP and NF-κB.

Abbreviations: CBP, CREB-binding protein; cdk, cyclin dependent kinase; HK, high potassium; LK, low potassium; PP2A, protein phosphatase 2A,

Key Words and Phrases: CBP, NF-κB, apoptosis, neuronal survival, depolarization, phosphatase 2A

Introduction

Apoptosis plays a critical role in the development of the mammalian nervous system serving to regulate neuronal numbers and to rid the nervous system of unwanted or superfluous neurons. In the mature nervous system, inappropriate apoptosis is implicated as an underlying defect in a variety of neurodegenerative diseases and following stroke or traumatic head-injury (for review, Mattson, 2000; Yuan and Yankner, 2000). Much attention has, therefore, been focused on understanding the intracellular signaling pathways that promote or inhibit neuronal apoptosis. A number of recent studies have provided evidence that NF-κB, a transcription factor involved in disparate processes such as inflammation, and growth and development, also plays an important role in the regulation of apoptosis in neurons and non-neuronal cells (reviewed in Ghosh et al., 1998, Karin and Ben-Neriah, 2000; Mattson et al., 2000). While some studies performed in neuronal systems have found NF-κB to be associated with apoptosis, many other studies have shown that NF-κB protects neurons from a variety of death-inducing stimuli (Lin et al., 1995; Hemanoue et al., 1998; Lin et al., 1998; Maggirwar et al., 1998; Qin et al., 1998; Taglialatela et al., 1998; Cheema et al., 1999; Kaltschmidt et al., 1999; Glazner et al., 2000; Daily et al., 2001; Yu et al., 2000).

In mammalian cells, there are five NF-κB proteins, p50, p52, p65 (RelA), RelB and c-Rel. Functional NF-κB is composed of homodimers and heterodimers of these proteins, typically p65: p50, which are held in the cytoplasm by the inhibitory IκB protein. In most cases, activation of NF-κB is mediated by the phosphorylation of IκB, which targets it for degradation thus permitting NF-κB to translocate to the nucleus and activate gene transcription (Ghosh et al., 1998, Karin and Ben-Neriah, 2000). Although

this is the best characterized mechanism of NF-κB activation, recent work has shown that NF-κB activity can also be regulated by IκB-independent mechanisms. For example, phosphorylation of p65 by kinases such as PKA and casein kinase increase its transcriptional activity (Naumann and Scheidereit, 1994; Diehl et al., 1995; Wang and Baldwin, 1998; Zhong et al., 1998; Anrather et al., 1999). Interaction with transcriptional coactivators such CREB-binding protein (CBP) and the paralogous protein p300 also increases NF-κB activity (Perkins et al., 1997; Merika et al., 1998).

CBP/p300 function as transcriptional coactivators by linking cellular activators (such as NF-κB, CREB, p53, c-fos, c-jun, c-myb, and MyoD) to components of the basal transcription machinery (for reviews, Snowden et al., 1998; Giordano and Avantaggiati, 1999; Goodman and Smolik, 2000; Vo and Goodman, 2001; Chan and La Thangue, 2001). Because CBP/p300 is capable of interacting with a wide range of transcription factors it may play an important role in the integration of diverse signaling pathways. It has been proposed that CBP/p300 may be present in limiting amounts within the nucleus (Wadgaonkar et al., 1999; Webster and Perkins, 1999). This taken together with the finding that some transcription factors bind to overlapping regions of CBP/p300 has led to the idea that a constant competition exists among transcription factors for binding to CBP and that depending on which factor binds CBP, different biological responses may result. For many transcription factors including NF-κB, CREB, p53 and c-jun, binding to CBP requires phosphorylation of the transcription factor (Giordano and Avantaggiati, 1999; Goodman and Smolik, 2000, Vo and Goodman, 2001). In comparison with the large amount of analysis performed on the binding of transcription factors to CBP / p300 and the effect of these interaction on the activities of different

transcription factors, little attention has been placed on how the activity of CBP / p300 is itself regulated. It is also noteworthy, that although often regarded as functional homologs, it is now known that CBP and p300 regulate distinct target genes (Yao et al., 1998). This along with the observation that p300, but not CBP, is involved in retinoic acid differentiation and cell-cycle arrest, and the prenatal death of mice lacking either CBP or p300 indicates that these two proteins have at least some non-overlapping functions which are critical for embryogenesis (Tanaka et al., 1997; Kawasaki et al., 1998, Yao et al., 1998).

We have been examining the role of NF-κB in the regulation of apoptosis in cultured cerebellar granule neurons. These neurons undergo apoptosis when shifted from medium containing serum and depolarizing concentrations of potassium (high K+ medium, HK) to medium containing low potassium (LK) (D'Mello et al., 1993). We recently reported that NF-κB is involved in HK-mediated survival of granule neurons (Koulich et al., 2001). Interestingly, however, neither the levels of the five NF-κB proteins nor those of IκB-α and IκB-β were altered in neurons primed to undergo apoptosis, implicating uncommon mechanisms in the regulation of NF-κB activity in these neurons. In this study, we show that p65 interacts with CBP in healthy granule neurons. The decrease in NF-κB transcriptional activity induced by LK-treatment is accompanied by a reduction in the interaction between p65 and CBP, a change associated with CBP hyperphosphorylation. We present results consistent with the possibility that CBP hyperphosphorylation is caused by the inactivation of a PP2A-related phosphatase. This alteration leads to reduced CBP-p65 interaction resulting in decreased NF-κB activity and consequently, to cell death.

Materials and Methods

Materials. Unless specified otherwise, all chemicals were purchased from Sigma Chemicals. Okadaic acid, staurosporine, endothall and cantharidin were purchased from Calbiochem. Unless specified otherwise, all antibodies used were purchased from Santa Cruz Biotechnology, Inc. The PP2A, PP4, and PP6 antibodies were the kind gift of Dr. Brian Wadzinski (Vanderbilt University).

Cell culture and treatments. Granule neuron cultures were obtained from dissociated cerebella of 7-8 day old rats as described previously (D'Mello et al., 1993). Cells were plated in Basal Eagle's Medium with Earles salts (BME) supplemented with 10% fetal bovine serum (FBS), 25 mM KCl, 2 mM glutamine (Gibco-BRL), and 100 ug/ml gentamycin on dishes coated with poly-L-lysine in 24-well dishes at a density 1.0×10^6 cells/well, 1.2×10^7 cells/60mm dish, or 3.0×10^7 cells/100 mm dish. Cytosine arabinofuranoside (10 uM) was added to the culture medium 18 - 22 h after plating to prevent replication of non-neuronal cells.

Unless indicated otherwise, cultures were maintained for 6 - 7 days prior to experimental treatments. For this, the cells were rinsed twice and then maintained in low K^+ medium (serum-free BME medium, 5 mM KCl) or high K^+ medium (serum-free BME medium, supplemented with 20 mM KCl.

Unless indicated otherwise in the figure legends, treatment of cultures with pharmacological inhibitors was initiated 15 min prior to rinsing and was maintained through the subsequent incubation in LK or HK medium.

Neuronal survival. Neuronal survival was quantified by the MTT assay as described by Kubo et al.,1995. Briefly, the tetrazolium salt MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolim bromide] was added to the cultures at a final concentration of 1 mg/ml, and incubation of the culture was continued in the CO_2 incubator for a further 30 min at 37^0C. The assay was stopped by adding lysis buffer [20% SDS in 50% N,N-dimethyl formamide , pH 4.7]. The absorbance was measured specrophotometrically at 570nm after an overnight incubation at room temperature. The absorbance of a well without cells was used as background and subtracted.

Results obtained using the MTT assays were confirmed using the fluorescein-diacetate method for quantification of cell viability, as previously described (D'Mello et al., 1993). Data are presented as mean +/- standard deviation. Statistical analysis was performed using ANOVA and Student-Neuman-Keuls' test.

Western blotting. For whole-cell lysates, the culture medium was discarded, the neurons washed twice with ice-cold phosphate-buffered saline (PBS), and lysed in sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis buffer [62.5 mM Tris-Cl, pH 6.8, 2% SDS, 5% glycerol, 1% 2-mercaptoethanol, and bromophenol blue]. Following heating at 95^0 for 5 min, proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membrane (PVDF; Bio Rad). After staining with Ponceau S (Sigma) to verify uniformity of protein loads / transfer, the membranes were analyzed for immunoreactivity. Incubation with primary antibodies was performed overnight at 4^0C and with secondary

antibodies for 1h at room temperature. Immunoreactivity was developed by enhanced chemiluminescence (ECL; Amersham) and visualized by autoradiography.

The following commercial primary antibodies were used: p65 (sc-372 or sc-372-G), CBP (sc-369 and sc-583), p300 (sc-584 or sc-585), and c-jun (sc-1694). All antibodies were used at a1:1000 dilution. Secondary antibodies were peroxidase-conjugated goat anti-rabbit IgG (sc-2004; 1:10,000) and anti-goat IgG (sc-2020; 1:5,000).

Immunoprecipitation. 100mm dishes of 7-8-day old neurons were washed twice with warm BME medium containing either 25mM KCl (HK) or 5mM KCl (LK) and incubated for 6h in the CO_2 incubator at 37^0C in the same medium. Cells were washed twice with ice-cold 1XPBS and lysed on ice in ice-cold NP-40 buffer (150mM NaCl, 1% Triton X-100 (or NP-40), 50mM Tris (pH 7.2 at 4°C), 1mM Na3VO4, 1mM NaF, 1mM PMSF, 20ul/ml protease inhibitors cocktail (Sigma)). The lysates were centrifuged for 10 min at 10,000 rpm at 4°C. Supernatants were incubated overnight with primary antibody (1.5 - 2ug) and then for 2h with 20ul Protein A/G PLUS-Agarose (Santa Cruz Biotech.). Immunoprecipitates were collected by centrifugation at 2,500 rpm for 5 min at 4°C, washed three times with NP-40 buffer, and pellets resuspended in electrophoresis sample buffer (187.5 mM Tris-HCl (pH 6.8 at 25°C), 6% SDS, 30% glycerol, 150mM DTT, 0.03% bromphenol blue), boiled for 4 min and subjected to SDS-polyacrylamide gel electrophoresis. Immunoprecipitated proteins were transferred to PVDF membrane by electrophoresis and Western blotting performed as described above.

Analysis of endogenous CBP phosphorylation. 60mm dishes of 7-8-day old neurons were washed twice with warm, phosphate-free DMEM (Gibco) containing 25mM KCl and incubated overnight in the same medium. The cultures were then incubated for 6h in medium containing [^{32}P] orthophosphate (300uCi/mL; ICN) in either 25mM (HK medium) or 5mM KCl (LK medium). Following lysis in ice-cold RIPA buffer (50mM Tris (pH 7.2 at 4°C), 150mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 1mM Na3VO4, 1mM NaF, 1mM PMSF, 1mM EDTA, 20ul/mL protease inhibitors cocktail (Sigma), the lysates were subjected to immunoprecipitation as described above and proteins separated on SDS-PAGE gel. After electrophoretic transfer to PVDF membrane, labeled proteins were visualized by autoradiography.

When inhibitors of kinases or phosphatases were used, they were added at the time of switching of neurons to the labeling medium.

Plasmid construction and expression of bacterially produced GST-CBP proteins. The full-length human CBP cDNA was a kind gift from Dr. Richard H. Goodman (Oregon Health Sciences University). The cDNA inserts for GST- CBP(300-449), GST-CBP(1662-2440), and GST-CBP(2187-2440) were generated by PCR using the full length CBP cDNA as template. The oligonucleotides used were as follows:
GST- CBP(300-449): GCCCCCGATCCCATATGCCCAACATGGGTCAACAGCC, and
CGCCTCGAGAGCTGGAGATCCCAGGATGGTTTG
GST-CBP(1662-2440): GGATCCGCCTTCCTCACCCTGGCCAGG, and
CTCGAGCTACAAACCCTCCACAAACTTTTC

GST-CBP(2187-2440): GGATCCCGAGAAATGGTGAGGAGGCAGCTG, and CTCGAGCTACAAACCCTCCACAAACTTTTC.

The purified PCR products were digested with *Bam*HI and *Xho*I and ligated in-frame into pGEX-4T3 (Amersham-Pharmacia Biotechnology), previously cut with the same enzymes. Sequences of all constructs were confirmed. The CBP pGEX-4T3 constructs were transformed into *Escherichia coli* BL21 (DE3) PlyS (Novagen). Cultures (250ml) of E.coli were grown to an optical density at 600 nm of 0.4 to 0.6 and induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3h. Cells were pelleted, resuspended in buffer A (20 mM HEPES [pH 7.9], 400mM NaCl, 5 mM dithiothereitol [DTT], 50 mM mannitol, 10 mM sodium ascorbate, 10% glycerol, 0.1 mM EDTA, 0.1% Nonidet P-40 [NP-40]; 1 Mm phenylmethylsufonyl fluoride [PMSF]), mildly sonicated, and centrifuged. Following fractionation through Q-Sepharose columns with a 0.1 to 0.5 M NaCl to remove contaminating DNA, the supernatant (flow through) was incubated with 0.5 ml glutathione agarose matrix (Amersham-Pharmacia Biotechnology) for 2h at 4^0C . The matrix was washed four times with buffer A and two times with buffer B (50 MM Tris [pH 8.0],, 120 mM NaCl, 0.5% NP-40, 5 mM DDT, 1 mM PMSF). These fusion proteins were eluted off matrix by 5 mM glutathione and then were dialyzed overnight against phosphate-buffered saline (PBS; pH 7.4) at 4^0C.

In vitro kinase assay of GST-CBP proteins. Bacterially expressed GST-CBP proteins were bound to glutathione agarose matrix (Amersham-Pharmacia Biotech.) for 1 h at 4 ^{0}C, and then incubated with whole cell lysates from LK and HK treated cultures in kinase reaction buffer (50 mM Tris [pH 7.4], 5mM MnCl2, 5 mM DTT). The whole cell

lysates were generated from cultures plated in 100 mm dishes (30 X 10^6 cells/dish) and lysed in a volume of 250 ul. The kinase assay was performed in the kinase reaction buffer with addition of 4 μM ATP, 10μ Ci of [γ-^{32}P] ,5 mM NaF, 1 mM sodium orthovanadate, 40 μM $MgCl_2$ a protease inhibitor cocktail (Sigma), and 1mM PMSF at 30^0C for 30 min. The matrix was pelleted by centrifugation , washed three times with PBS buffer. After addition of 6x SDS sample buffer to the pellet, the samples were heated at 95^0C for 5 min. The proteins were resolved with SDS-10% polyacrylamide gels followed by autoradiography.

Metabolic labeling and pulse-chase assay. 60mm dishes of 7-8-day old neuronal cultures were deprived of cysteine (Cys) and methionine (Met) for 15 min and then incubated in either HK or LK Cys/Met free DMEM (Gibco-BRL) with addition of ^{35}S Cys/Met (0.2mCi/ml; ICN). After a 20 min pulse phase, the cultures cells switched to either HK or LK BME medium for 4h. The chase phase was terminated by lysis of cells with NP-40 buffer (150mM NaCl, 1% Triton X-100 (or NP-40), 50mM Tris (pH 7.2 at 4°C), 1mM Na3VO4, 1mM NaF, 1mM PMSF, 20ul/ml protease inhibitors cocktail (Sigma)) and followed by immunoprecipitation as described above.

Analysis of NF-κB activity. Neurons were plated on 24 well dishes and transfected with either pNF-κB-Luc (Clontech Lab.) or pGL3-Basic (Promega) vectors on the day 5 after plating using the calcium phosphate method, as described by Koulich et al., 2001. The following morning, the neurons were switched to either HK or LK for 6h. The cells were lysed and used for quantification of luciferase activity using the Luciferase Assay

System and the protocol supplied by the manufacturer (Promega). Luciferase activity was measured using a luminometer (Turner Designs, Model 20).

Results

NF-κB activity is reduced following K+ deprivation

Cultured cerebellar granule neurons undergo apoptosis when switched from HK-medium to medium containing LK (D'Mello et al., 1993). In this paradigm, commitment to apoptosis occurs within 6 h after the switch to LK although cell death itself begins only after about 16 h (Galli et al., 1995, Schulz et al., 1996, Nardi et al., 1997; Borodezt and D'Mello, 1997). We have previously shown that LK-induced apoptosis can be mimicked by inhibitors of NF-κB activity implicating this factor in the survival effects of HK (Koulich et al., 2001). The finding that the DNA-binding activity of a p65-containing complex is reduced within 4 h of LK-treatment (Koulich et al., 2001) supports this idea. Moreover, overexpression of p65, reduces LK-induced cell death while overexpression of IκB-α causes apoptosis even in HK medium (Koulich et al., 2001). Surprisingly, however, neither the endogenous levels of the five NF-κB proteins, nor that of IκB-α or IκB-β, are altered by LK-treatment (Koulich et al., 2001). Since DNA-binding assays are only an indirect indication of transcriptional activity, we investigated whether LK-treatment did, in fact, reduce NF-κB activity. Neuronal cultures were transfected with a plasmid containing four tandem copies of the NF-κB consensus sequence upstream of a minimal promoter fused to a luciferase reporter and then switched to HK or LK medium. As shown in Figure 1, a 6 h switch to LK medium resulted in an approximately 80% reduction in NF-κB transcriptional activity.

Interaction of p65 with CBP is reduced following K+ deprivation

Although NF-κB activation is generally mediated by IκB degradation, the transcriptional activity of some NF-κB proteins, such as p65, can also be stimulated within the nucleus by interaction with the transcriptional coactivator CBP/p300 (Perkins et al., 1997; Merika et al., 1998). As a step towards determining whether p65 activity was regulated by interaction with CBP/p300, we examined whether these transcriptional coactivators were expressed in granule neuron cultures. Although present in HEK293 and 3T3 cell-lines, the expression of p300 was barely detectable in granule neurons by Western blot analysis (Figure 2). In contrast, CBP expression is clearly detectable. The expression of CBP, however, was similar under HK and LK conditions (Figure 2). Because of its robust expression levels, we focused our attention on CBP only.

To investigate the possibility that p65 interacts with CBP, we immunoprecipitated p65 from cells exposed to HK or LK treatment, and subjected the immunoprecipitate to Western blot analysis using a CBP antibody. As shown in Figure 3A, p65 does interact with CBP in granule neurons. More interestingly, the level of interaction was substantially reduced by LK-treatment. A similar result was obtained when CBP-immunoprecipitates were subjected to Western blotting using a p65 antibody (Figure 3B).

It is believed that CBP is present in limiting amounts within cells and that different proteins compete for binding to CBP (Wadgaonkar et al., 1999; Webster and Perkins, 1999). To examine whether the reduced binding of p65 with CBP is accompanied by an increase in the binding between CBP and another protein, we metabolically labeled

proteins in neuronal cultures treated with HK and LK medium using ^{35}S-methionine-cysteine and visualized CBP-interacting proteins following CBP immunoprecipitation. As shown in Figure 4 A, LK-treatment leads to increased interaction between CBP and a protein of approximately 43 kDa molecular weight. One protein that is of this size, and which is known to interact with CBP, is the pro-apoptotic transcription factor c-jun (Arias et al., 1994; Bannister et al., 1995). Moreover, it has been reported that c-jun and p65 compete for binding to CBP in PC12 cells (Maggirwar et al., 2000). We, therefore, examined whether c-jun interacts with CBP in granule neurons and whether this interaction is increased by LK-treatment. As shown in Figure 4 B, while interaction of c-jun with CBP is detectable, LK-treatment does not change the level of interaction.

Phosphorylation of CBP is increased by LK

Previous studies have shown that p65 interaction with CBP depends on the phosphorylation of p65 (Zhong et al., 1998). Using *in vivo* labeling with [^{32}P] orthophosphate, we have found that p65 is modestly phosphorylated in granule neurons but that the level of phosphorylation is not changed by LK treatment (Koulich et al., 2001). Since p65 phosphorylation was not altered by LK treatment and because CBP expression was not changed, we examined if the alteration in p65-CBP interaction was due to CBP phosphorylation. As shown in Figure 5 A and B, CBP phosphorylation is detectable normally but is increased by LK-treatment.

To gain insight into the mechanism of CBP hyperphosphorylation we analyzed the ability of various kinase inhibitors to inhibit CBP hyperphosphorylation. As shown in Figure 5B, the increase in CBP phosphorylation can be prevented by high doses of the

broad-spectrum kinase inhibitor, staurosporine. To examine if phosphatases were also involved in regulating CBP hyperphosphorylation, we used cyclosporin A (a PP2B inhibitor) as well as okadaic acid and cantharidin, two inhibitors highly selective for PP2A and the more recently discovered PP2A-related phospatases PP4 and PP6. While cyclosporin A had no effect, treatment with okadaic acid and cantaridin both caused CBP hyperphosphorylation in HK medium (Figure 5 B). Although okadaic acid and cantharidin are also known to inhibit PP1, they do so only at concentrations that are 10 - 50 fold higher than those used in this study. A similar hyperphosphorylation of CBP was observed in HK medium using endothall, another highly selective inhibitor of PP2A and related phosphatases (data not shown).

As a first step towards gaining insight into which PP2A or related phosphatase was involved in CBP hyperphosphorylation, we examined if PP2A, PP4, and PP6 were expressed in these neuronal cultures. As shown in Fig. 6A, the expression of all three of these phosphatases is easily detectable. We examined if any of these three phosphatases associated with CBP in granule neurons. As shown in Fig. 6B, PP6 but not PP2 or PP4 interacts strongly with CBP.

To localize where within CBP the LK-induced hyperphosphorylation occurred, we expressed cloned fragments of CBP as GST fusion proteins. The purified GST-CBP proteins were incubated with whole cell lysates from LK or HK treated neuronal cultures in the presence of [^{32}P]-ATP, and the ability of the lysates to phosphorylate the various CBP fragments was ascertained. As shown in Figure 7 B, a fragment spanning residues 1662 - 2440 of CBP was phosphorylated by both HK and LK lysates. The extent of phosphorylation was higher with LK lysates than that seen with HK lysates,

recapitulating *in vitro* the pattern of CBP hyperphosphorylation observed *in vivo*. In contrast, a fragment spanning residues 2187 - 2440 showed no phosphorylation, localizing the LK-induced site of hyperphosphorylation to the 525 aminoacid region between residues 1662 - 2187 (Figure 7 B). *In vitro* kinase analysis of another CBP fragment spanning residues 300 - 449 and containing the C/H1 motif was also efficiently phosphorylated (Figure 7 C). In this case, however, the extent of phosphorylation was similar in LK and HK conditions. To determine if phosphorylation of the C-terminus fragment was sensitive to okadaic acid and cantharidin, these phosphatase inhibitors were added to cultures treated with HK medium. As shown in Figure 7 C, the higher phosphorylation of $CBP_{(1662-2440)}$ seen in LK is also observed with lysates from HK cultures treated with the okadaic acid and cantaridin. The inhibitors had no effect on the phosphorylation of the C/H1 containing fragment, $CBP_{(300-449)}$ (Figure 7 C).

More detailed analysis using in vitro kinase assays was performed on the region spanning residues 1662 - 2187 of CBP. As shown in Figure 7C, a region spanning residues 1662 - 1840 was sufficient to observe hyperphosphorylation in response to LK treatment and to treatment with okadaic acid or cantharidin. No difference in phosphorylation was seen in another fragment spanning residues 1840 - 2100 of CBP (not shown).

Agents that induce CBP hyperphosphorylation reduce p65-CBP association and cause cell-death.

If CBP hyperphosphorylation was causally involved in promoting cell death, a prediction that could be made is that agents that induce CBP hyperphosphorylation

would cause cell death even under survival-promoting culture conditions. On the other hand, treatment with agents that inhibit CBP hyperphosphorylation would be expected to prevent cell death even in the absence of survival-promoting factors. To examine this we treated neuronal cultures in HK medium with okadaic acid or cantharidin, and in LK-medium with staurosporine. As shown in Figure 8A and B, high doses of staurosporine, which inhibit CBP hyperphosphorylation, also inhibit LK-induced apoptosis. Similarly, okadaic acid, cantharidin, and endothall, all of which induce CBP hyperphosphorylation, promote apoptosis in HK medium (Figure 8A and C). Not unexpectedly, treatment with cyclosporin A (which has no effect on CBP phosphorylation) has no effect on HK-mediated neuronal survival.

To determine whether the reduced association between CBP and p65 observed following LK treatment was also due to CBP hyperphosphorylation, we treated neuronal cultures with okadaic acid or cantharidin. As shown in Figure 8, treatment with these CBP-hyperphosphorylating agents leads to a reduction in CBP-p65 interaction even in HK medium (Figure 9).

Discussion

Several laboratories have shown that activation of NF-κB is involved in the inhibition of apoptosis in neurons and non-neuronal cells (for review, Mattson et al., 2000). In general, the activation of NF-κB is mediated by the translocation of NF-κB into the nucleus resulting from the degradation of its cytoplasmic inhibitor IκB. As demonstrated in many other cell types, overexpression of p65 inhibits apoptosis in cerebellar granule neurons induced by LK-treatment while overexpression of IκB-α causes apoptosis even in HK (Koulich et al., 2001). In contrast to a majority of neuronal and non-neuronal systems, however, in granule neurons the reduced NF-κB activity during apoptosis is not accompanied by any change in the expression of the five NF-κB proteins. Similarly, the levels of IκB-α or IκB-β remain unchanged by LK-treatment (Koulich et al., 2001). Thus, the molecular basis for the decreased DNA-binding activity of p65 observed in LK-treated neurons has been unclear. We now show that p65 interacts with the transcriptional coactivator CBP in healthy neurons, and that this interaction is reduced when neurons are switched from HK to LK medium. Previous studies performed in other systems have shown that interaction with CBP increases, and may even be necessary for the transcriptional activity of p65 (Zhong et al., 1998).

Using *in vitro* assays, a previous study by Zhong et al. 1998, has shown that the interaction of p65 with CBP depends on the phosphorylation of p65 at serine 276. Phosphorylation at serine 276 disrupts an intramolecular interaction between the N-terminal region and the C-terminal region of p65 that unmasks the binding sites for association with CBP (Zhong et al., 1998). In granule neurons, we find that p65 is equally phosphorylated in HK and LK treatment. This level of p65 phosphorylation may

permit it to interact with CBP. Our results indicate that the reduced level of interaction between p65 and CBP in LK may be regulated by the extent of CBP phosphorylation. Although previous studies have shown that CBP and p300 can be phosphorylated at several sites (Blom et al., 1999), only two of these sites (Ser89 by PKC and Ser301 by CaM Kinase IV) have been identified so far (Yuan and Gambee, 2000; Impey et al., 2002). Both of these sites lie within the N-terminus region of CBP/p300. Using in vitro kinase assays, we have mapped the LK-regulated phosphorylation site to a region spanning residues 1642 - 1840 of CBP. It is possible that phosphorylation of CBP results in a conformational change thus reducing its affinity for p65. Also possible is that CBP phosphorylation increases its affinity for a pro-apoptotic protein, the binding of which causes the displacement of p65 from CBP. *In vivo* labeling experiments showed a robust increase in the interaction between CBP and a protein of approximately 43 kDa size. One protein of that size that is known to interact with CBP and which is necessary for LK-mediated apoptosis in granule neurons is c-jun (Miller et al., 1997; Watson et al., 1998; Shimoke et al., 1999). Although we have found that c-jun associates with CBP in granule neurons, the level of interaction is not increased in LK- medium arguing against the possibility that c-jun competes with p65 for binding to CBP.

Our results indicate that a PP2A-related phosphatase may normally keep CBP in an underphosphorylated state and consequently maintain neuronal survival. Reduced activity of such a phosphatase would cause CBP hyperphosphorylation. Consistent with this possibility is the finding that inhibitors of PP2A-like phosphatases induce CBP hyperphosphorylation and cause cell death. The unavailability of specific inhibitors or assays for the measurement of activity for each these PP2A-like phosphatases makes it

difficult to determine which of these highly-related phosphatases is responsible for dephosphorylating CBP. Our finding, however, that PP6 associates with CBP implicates it as the member involved in CBP hyperphosphorylation.

CBP and p300 were originally discovered as transcriptional coactivators (for reviews, Giordano and Avantaggiati, 1999; Goodman and Smolik, 2000, Vo and Goodman, 2001). More recent studies have implicated these proteins in other biological processes such as differentiation and cell-cycle control, protein stability, DNA repair, and tumor suppression (Grossman et al., 1998; Ito et al., 2001; Hasan et al., 2001). How these diverse actions of CBP/p300 are regulated is presently not known. It is known that CBP/p300 can be phosphorylated, although the effect of phosphorylation on the activity of these proteins, or the biological consequence of such phosphorylation, is far from clear. In this report, we show that CBP hyperphosphorylation is associated with the induction of apoptosis. We find that treatment with staurosporine, which inhibits CBP hyperphosphorylation, also inhibits LK-induced apoptosis. The antiapoptotic effect of staurosporine is particularly intriguing given that this inhibitor has been the prototypical apoptosis-inducing agent in a variety of cell types, including granule neurons (Taylor et al., 1997). We find that the antiapoptotic effect of staurosporine is only observed at very high doses (≥1 mM). The need for such high concentrations implies that the kinase responsible for apoptosis in granule neurons is only weakly inhibited by staurosporine. Moreover, the inhibition of this pro-apoptotic kinase can override the protective effect of a kinase(s) that is necessary for the survival of many cell types and which is inhibited by moderate concentrations of staurosporine at which this drug causes cell-death. The identity of the pro-apoptotic kinase that

hyperphosphorylates CBP and is inhibited by staurosporine is not known. It is noteworthy that CBP/p300 phosphorylation has found to be increased during cell progression and some reports have suggested that kinases necessary for cell-cycle progression, such as the cyclin-dependent kinase-2, interact with and may be involved in phosphorylating CBP/p300 (Yaciuk and Moran, 1991, Perkins et al., 1997, Ait-Si-Ali et al., 1998, Xu et al., 1998). Perkins et al. (1997) have shown that both p65 and cyclin E-cdk2 bind CBP/p300. Additionally, overexpression of the cdk inhibitor p21 or a dominant-negative cdk2 inhibited CBP/p300 associated cdk2 activity, which resulted in a stimulation of NF-κB activity (Perkins et al., 1997). Work by Padmanabhan et al. (1999) has shown that treatment of cerebellar granule neurons with olomoucine and other pharmacological inhibitors of cdk2 prevents LK-induced apoptosis raising the possibility that these inhibitors exert their antiapoptotic activity by inhibiting cdk2-mediated CBP hyperphosphorylation. Interestingly, the domain of CBP that is regulated in neurons by LK (residues 1662-1840) is within the region proposed by Ait-Si-Ali et al. (1998) to contain the cdk2 phosphorylation site. It is tempting to speculate that CBP hyperphosphorylation is associated with an abortive attempt of these neurons to traverse the cell-cycle. CBP/p300 phosphorylation has found to be increased during cell cycle progression (Yaciuk and Moran., 1991, Ait-Si-Ali et al., 1998, Xu et al., 1998) and several studies have provided evidence suggesting that components of the cell cycle machinery participate in the activation of apoptosis in neurons (Freeman et al., 1994, Park et al., 1997, Park et al., 1998, Padmanabhan et al., 1999).

In summary, we report that in cerebellar granule neurons, the activity of NF-κB is regulated by an uncommon mechanism involving interaction with CBP. This interaction

and hence NF-κB activity is reduced by CBP hyperphosphorylation, which contributes to cell death. Although phosphorylation of CBP has previously been reported, the association of this modification with the induction of apoptosis is, to our knowledge, novel. Our results are consistent with the involvement of PP6 in maintaining CBP in a underphosphorylated state under HK conditions.

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Legends

Figure 1: NF-κB transcriptional activity is reduced in LK.

Neuronal cultures were transfected with pNF-κB-Luc (NF-κB) vector, or a similar plasmid, pGL3-Basic, lacking the NF-κB elements (Control) on day 5 of neuronal culture. On day 6, the cultures were switched to LK or HK medium for 6h and luciferase assays performed. Results shown are from two independent experiments performed in triplicate.

Figure 2: CBP is expressed in cerebellar granule neurons, but its expression is not changed by LK treatment.

Whole cell lysates from granule neurons treated for 6h with HK or LK medium were analyzed by Western blotting using p300 antibody. Also included were lysates from the 3T3 and HEK293 cell-lines. While p300 is abundant in cell lines, its expression is barely detectable in neuronal extracts. The same blot was reanalyzed using a CBP antibody. Although CBP expression is clearly detectable, its level is similar in HK and LK treated cultures.

Figure 3: p65 and CBP interact in granule neurons and the level of interaction is reduced by LK treatment.

Neuronal cultures were treated with HK or LK medium for 6 hrs. The cells were then lysed. A portion of the lysates were used kept aside and the rest subjected to immunoprecipitation with p65 or CBP antibodies. The immunoprecipitate as well as the aliqouts of whole cell lysates was subjected to Western blotting.

A Lysates from HK and LK treated cells were immunoprecipitated with p65 antibody (lanes - $IP_{(p65)}$) and the immunoprecipitate subjected to Western blotting using a CBP antibody. The amount of p65 interacting with CBP is reduced in LK medium. Lanes marked WCL contain aliquots of whole cell lysates taken prior to p65 immunoprecipitation.

B. Lysates from HK and LK treated cells were subjected to immunoprecipitation with a CBP antibody ($IP_{(CBP)}$). The immunoprecipitates were then subjected to Western blotting with a p65 antibody along with aliquots of unimmunoprecipitated whole cell lysates (WCL). The asterisk points to a non-specific band recognized by the p65 antibody.

Figure 4: Interaction between CBP and a ~43 kDa protein other than c-jun increases following LK treatment.

A. Increased binding of an ~43 kDa protein to CBP in LK. Neuronal cultures were labeled with ^{35}S-methionine-cysteine for 30 min in HK or LK medium and then switched to the same medium containing cold methionine and cysteine for 4 h. The lysates were immunoprecipitated with a CBP antibody and immunoprecipitates subjected to SDS-PAGE. CBP-interacting proteins were visualized by autoradiography. One of the arrows points to an ~ 43 kDa protein that is pulled down by the CBP antibody and the binding of which is higher in LK. The other arrow points to a 65 kDa protein, which we have identified as p65.

B. Interaction between c-jun and CBP is not altered in LK. Neuronal cultures were treated with HK or LK medium for 6 hrs. The cells were then lysed and lysates

subjected to immunoprecipitation with c-jun or CBP antibodies. The immunoprecipitate was subjected to Western blotting along with aliquots of unimmunoprecipitated whole cell lysates.

Left panel: Lysates from HK and LK treated cells were immunoprecipitated with c-jun antibody (lanes - $IP_{(jun)}$) and the immunoprecipitate subjected to Western blotting along with whole cell lysates (lanes- WCL). A CBP antibody was used for the Western blot. No change is seen in the intensity of CBP.

Right panel: Lysates from HK and LK treated cells were immunoprecipitated with a CBP antibody (lanes - $IP_{(CBP)}$) and the immunoprecipitate subjected to Western blotting along with whole cell lysates (WCL). A c-jun antibody was used for the Western blot. The multiple c-jun bands seen in whole cell lysates from LK-treated cultures represents newly synthesized and phosphorylated forms of c-jun.

Figure 5: CBP phosphorylation can be regulated by the actions of a staurosporine-sensitive kinase and an okadaic acid / cantharidin -sensitive phosphatase.

Neurons were switched for 6h to HK or LK medium or to LK medium containing 1 uM staurosporine (LK + STS), HK medium containing 100 nM cantharidin (HK + Canth), 500 uM cyclosporin A (HK + Csp) or 20 nM okadaic acid (HK + OA) in the presence of [^{32}P] orthophosphate (300uCi/ml). Whole cell lysates were prepared and CBP immunoprecipitated. The extent of CBP phosphorylation was analyzed by SDS-PAGE and autoradiography. Lanes marked WCL contain whole cell lysate from cerebellar granule neurons that was loaded along with the products of the in vitro kinase reactions.

Figure 6. Analysis of PP2A, PP4, and PP6 expression patterns and ability to interact with CBP.

A. Expression of PP2A, PP4, and PP6. Expression of PP2A, PP4, and PP6 was analyzed by Western blotting in tissue and cell lysates. From l - r : Lysates from kidney, brain, the human Jurkat cell line, NIH3T3 cell line, and cerebellar granule neurons (treated with LK or HK medium for 6h).

B. Association with CBP. Cerebellar granule cell lysates were used for immunoprecipitation using antibodies against CBP, PP2A, PP4A and PP6. The immuoprecipitates were separated by PAGE and subjected to Western blot analysis using a CBP antibody. Lane labeled WCL contains unimmuoprecipitated whole cell lysates.

Figure 7: Analysis of LK-induced CBP hyperphosphorylation using GST-CBP fusion proteins.

A. Structure of CBP showing location of the cysteine-histidine rich C/H1, C/H2, and C/H3 regions and the histone acetyltransferase (HAT) domain. The CBP fragments used in this study and the regions covered within these proteins are indicated.

B. CBP hyperphosphorylation occurs within C-terminus half of CBP. Bacterially-expressed GST-CBP proteins spanning regions between residues 1662 - 2442 and residues 2187 - 2442 were bound to glutathione agarose matrix, and incubated with whole cell lysates from LK or HK treated (6 h) neuronal cultures in a kinase reaction buffer in the presence of [^{32}P]-ATP. Following pelleting of matrix and boiling of the samples in SDS buffer, the proteins were resolved by SDS-PAGE and subjected to

autoradiography. The position of the 124 kDa GST-CBP protein spanning residues 1662 - 2442, and the 55 kDa GST-CBP protein spanning residues 2187 - 2442 are indicated by arrows. The 124 kDa CBP(1662 - 2442) protein is phosphorylated in both LK and HK conditions, although the level of phosphorylation is higher with LK lysates. In contrast, the 55 kDa CBP(2187 - 2442) is not phosphorylated.

C. *In vitro* hyperphosphorylation of GST-CBP is inhibited by PP2A inhibitors. GST-CBP proteins spanning regions between residues 1662 - 2442, residues 1662 - 1840, and residues 300 - 449 were used in *in vitro* kinase reactions using lysates from neuronal cultures treated for 6 h with LK medium, HK medium, or HK medium containing okadaic acid or cantharidin. Following completion of the kinase reaction, the samples were analyzed by SDS-PAGE and autoradiography as described above. The 44 kDa N-terminus C/H1 spanning CBP (300-449) protein is phosphorylated in both, LK and HK medium. The level of phosphorylation is, however, similar and is not changed by treatment with okadaic acid or cantharidin. In comparison, the two PP2A inhibitors cause an increase in the phosphorylation of 124 kDa CBP(1662 - 2442). Further analysis showed localized the phosphorylation to the region spanning residues 1662 - 1840.

Figure 8: Neuronal survival is regulated by phosphatases and kinases.
Neurons were switched to HK-medium containing phosphatase inhibitors or to LK-medium containing staurosporine. Neuronal viability was quantified 24 h later using the MTT assay. Results shown represent mean values and come from three experiments performed in duplicate.

A. Phase-contrast photomicrographs of cultures treated for 24 h with HK, LK, HK plus 10 nM okadaic acid, or LK plus 2uM staurosporine.

B. Quantification of cell viability following staurosporine treatment. Neuronal cultures were switched to LK medium containing staurosporine at 0, 0.01, 0.1, 1 and 2 uM concentrations. Control cells received HK-medium.

C. Quantification of cell viability following treatment with phosphatase inhibitors. Neurons were treated with LK medium, or HK medium containing 15 nM okadaic acid, 500 nm cantharidin, or 1 uM endothall. Control cells received HK-medium.

Figure 9: CBP hyperphosphorylation causes reduced p65 binding.

A. PP2A inhibitors reduce the association of CBP with p65. Neuronal cultures were treated with LK medium, HK medium, or HK medium containing okadaic acid (HK + Okad. Ac.) or cantharidin (HK + Canthar.) for 6 hrs. The cells were then lysed and lysates subjected to immunoprecipitation with p65 antibody . The immunoprecipitate was subjected to Western blotting using CBP. In the presence of okadaic acid or cantaridin, the elevated CBP-p65 association seen in HK, is reduced.

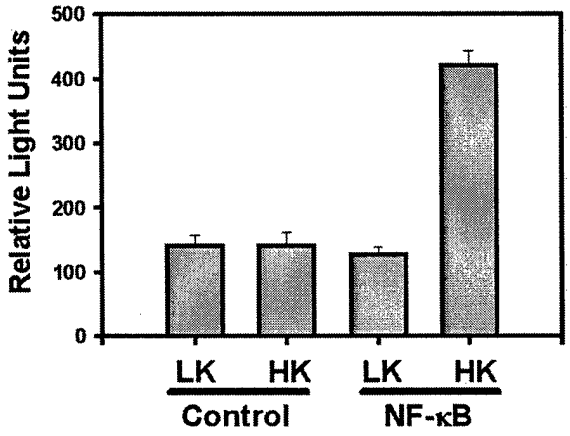

Relative Light Units
500
400
300
200
100
0
LK
HK
LK
HK
Control
NF-κB

FIGURE 1

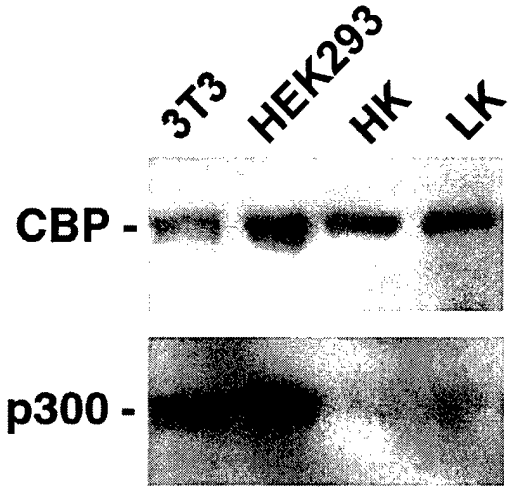


FIGURE 2

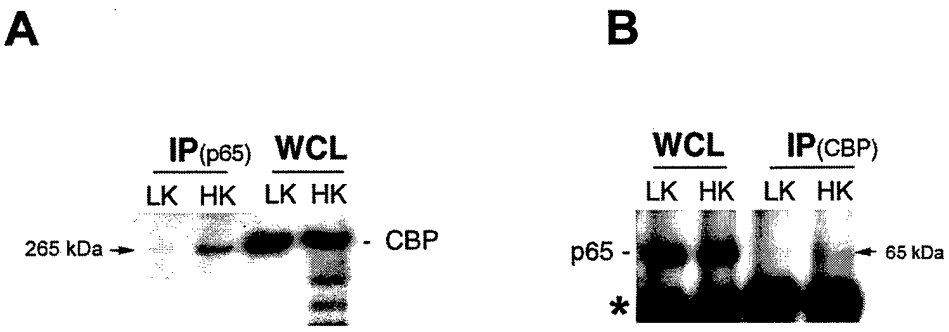
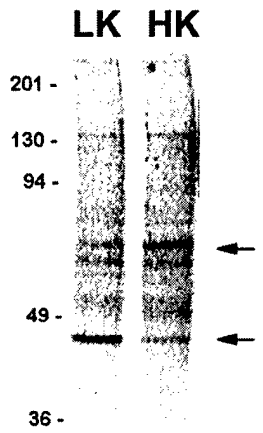

A
IP(p65)
WCL
LK HK LK HK
265 kDa
CBP
B
WCL
IP(CBP)
LK HK LK HK
p65
65 kDa
*

FIGURE 3

A


LK
HK
201 -
130 -
94 -
49 -
36 -

B

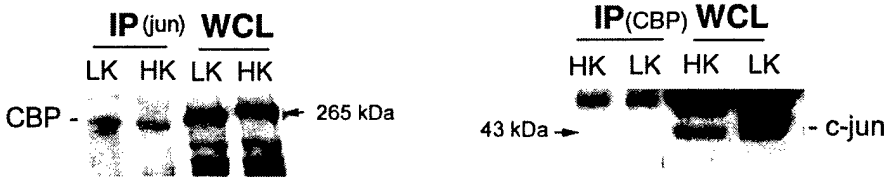

IP (jun)
WCL
LK
HK
LK
HK
CBP -
265 kDa
IP(CBP)
WCL
HK
LK
HK
LK
43 kDa
c-jun

FIGURE 4

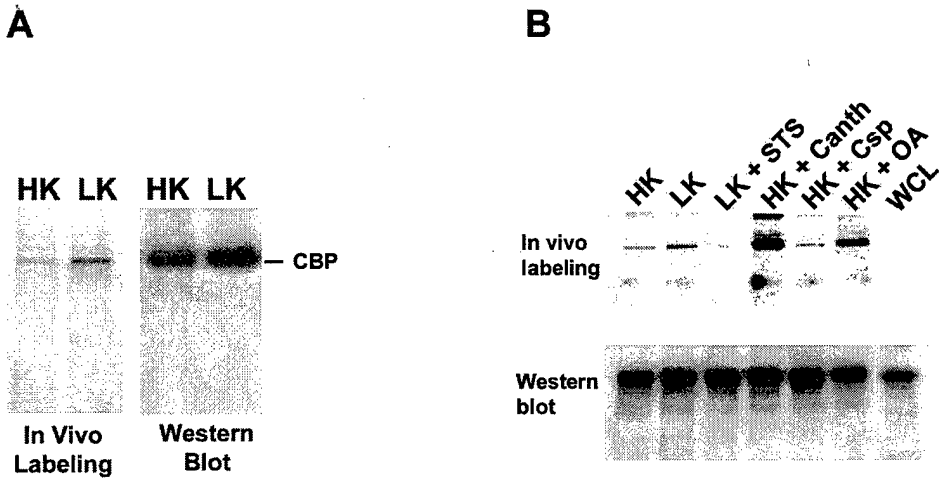
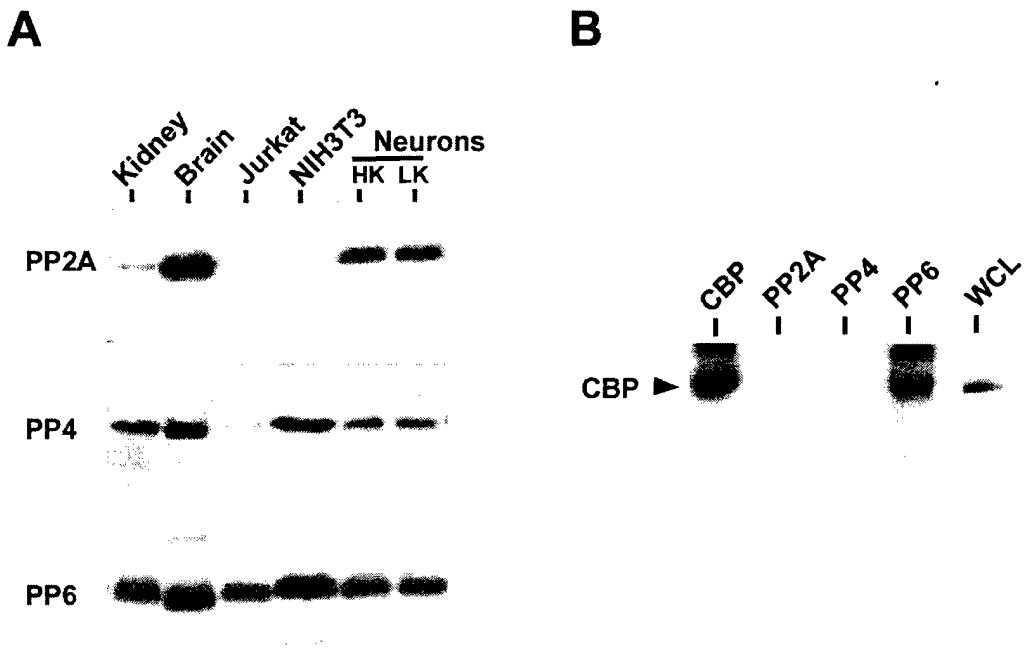

A
HK LK HK LK
CBP
In Vivo Labeling
Western Blot
B
HK
LK
LK + STS
HK + Canth
HK + Csp
HK + OA
WCL
In vivo labeling
Western blot

FIGURE 5


A
Kidney
Brain
Jurkat
NIH3T3
Neurons
HK
LK
PP2A
PP4
PP6
B
CBP
PP2A
PP4
PP6
WCL
CBP

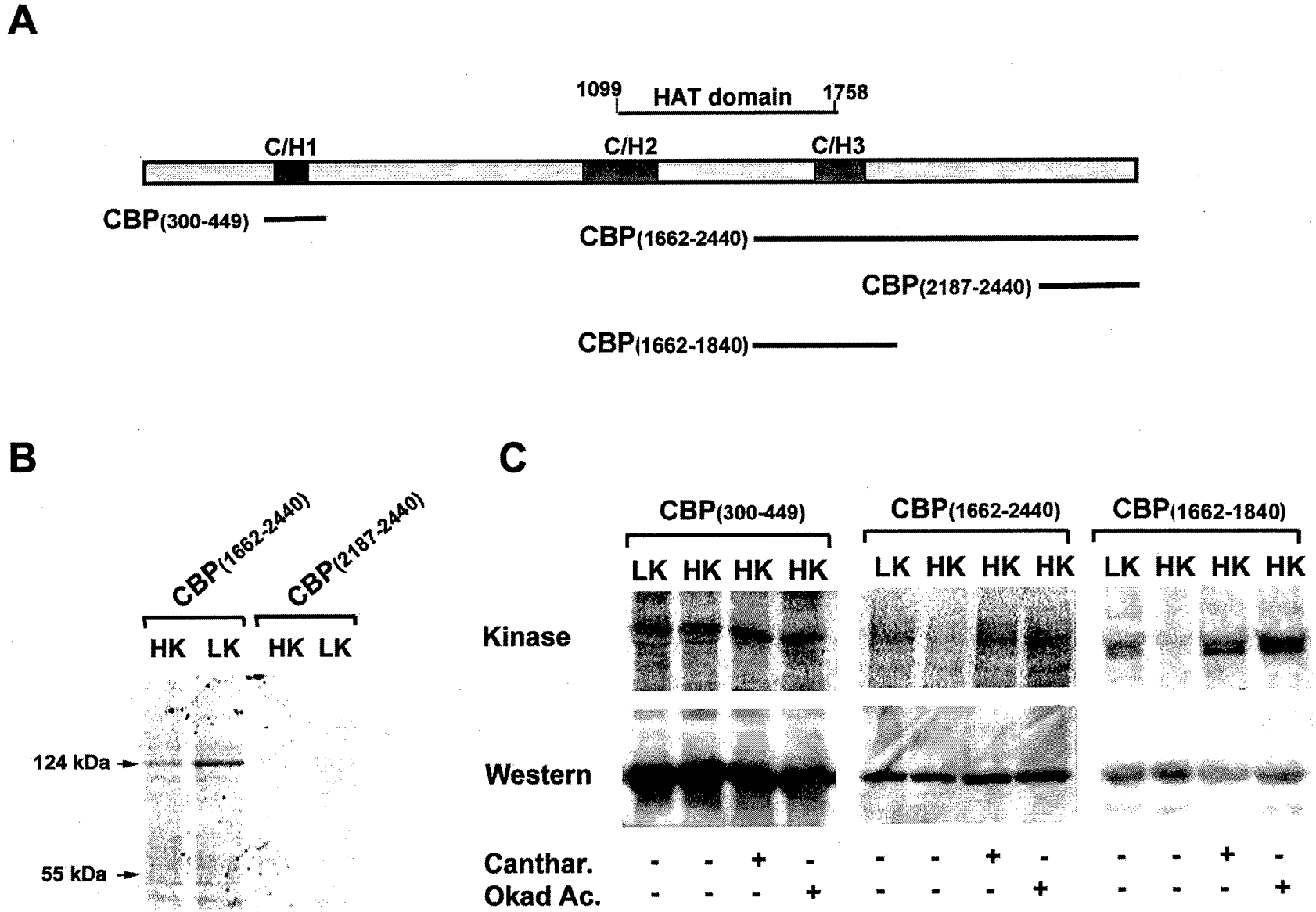
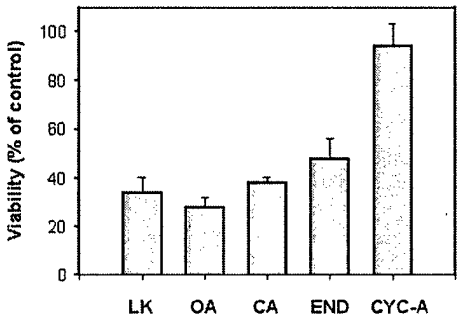
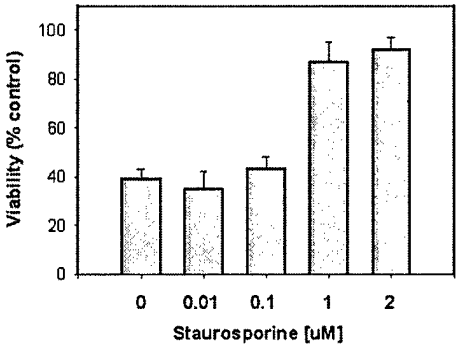

A
1099
HAT domain
1758
C/H1
C/H2
C/H3
CBP(300-449)
CBP(1662-2440)
CBP(2187-2440)
CBP(1662-1840)
B
CBP(1662-2440)
CBP(2187-2440)
HK LK HK LK
124 kDa
55 kDa
C
CBP(300-449)
CBP(1662-2440)
CBP(1662-1840)
LK HK HK HK
LK HK HK HK
LK HK HK HK
Kinase
Western
Canthar. - - + - - - + - - - + -
Okad Ac. - - - + - - - + - - - +

FIGURE 7

A


Viability (% of control)
100
80
60
40
20
0
LK
OA
CA
END
CYC-A

B


Viability (% control)
100
80
60
40
20
0
0
0.01
0.1
1
2
Staurosporine [uM]

C

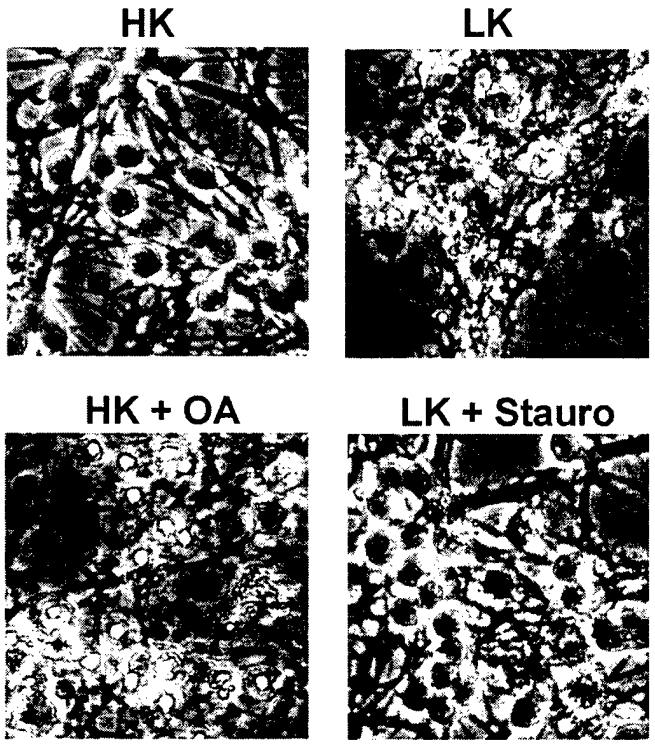

HK
LK
HK + OA
LK + Stauro

FIGURE 8

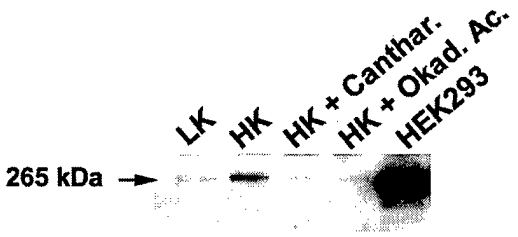


FIGURE 9